

IMPACT OF THE DNA METHYLTRANSFERASE INHIBITOR, 5-AZACYTIDINE, ON  
CHONDROCYTIC PHENOTYPE

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements  
for the degree of Doctor of Philosophy in VMS - Veterinary Clinical Medicine  
in the Graduate College of the  
University of Illinois at Urbana-Champaign, 2011

Urbana, Illinois

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## ABSTRACT

Epigenetics has been shown to be involved in cellular differentiation, aging and disease development. This research project had the objective of verifying whether epigenetics, more specifically DNA methylation, plays a role in chondrocyte gene regulation during osteoarthritis and cellular differentiation.

5-azacytidine (5-aza) is a DNA methyltransferase inhibitor that has been used to treat acute myelogenous leukemia due to its ability to cause global DNA demethylation and therefore activation of transcription of tumor suppressor genes that are commonly repressed by DNA methylation in this disease. We have used 5-aza to investigate whether decreased expression of collagen type II by articular chondrocytes in osteoarthritis could be relieved by 5-aza. Chondrocytes were isolated from articular cartilage obtained from humans undergoing total joint arthroplasty due to knee osteoarthritis. The cells were treated with 5-aza for 4 days and the expression of several chondrocytic genes were analyzed by Northern Blot and quantitative PCR analyses. The results showed that 5-aza upregulated several chondrocytic genes; however the methylation analyses of the collagen type II promoter showed that the collagen type II promoter is highly unmethylated. Other mechanisms for the 5-aza effects in articular chondrocytes such as, increase in expression of transcriptional activators, decrease expression of transcriptional suppressors, increase in expression of growth factors known to modulate the chondrocytic phenotype and activation of the DNA damage response with upregulation of p21 and p53 were investigated. 5-aza showed to increase the expression of TGF- $\beta$ 3 and its downstream mediator CTGF; however we did not investigate whether the upregulation of these genes were due to demethylation of their regulatory regions. Adenoviral over-expression of p53 and p21 did not mimic 5-aza effects in articular chondrocytes. Nonetheless, the results showed that 5-aza increases the expression of extracellular matrix genes critical for cartilage homeostasis in aged- and/or osteoarthritic chondrocytes.

Osteoarthritic chondrocytes undergo phenotypic changes that resemble hypertrophic differentiation. Collagen type X is one of the hypertrophic markers that are expressed by osteoarthritic chondrocytes. Zimmerman *et al.* (2008) demonstrated that collagen type X expression correlated with demethylation of specific CpGs in the

collagen type X promoter by mesenchymal stem cells undergoing chondrogenesis. We investigated whether increase in expression of collagen type X by osteoarthritic chondrocytes also was due to demethylation of the same specific CpGs. Articular cartilage was collected from humans undergoing total joint arthroplasty and categorized as normal or osteoarthritic based on its macroscopic appearance. The articular phenotype was assessed by expression of collagen type II and aggrecan and the hypertrophic markers collagen type X and alkaline phosphatase were also assessed by quantitative PCR. The methylation status of the collagen type X gene was analyzed by methylation sensitive restriction enzyme digestion followed by PCR. Our results showed that collagen type X expression was independent of demethylation of the specific CpG sites and that overall the collagen type X promoter is highly demethylated in articular chondrocytes.

We also have used 5-aza as mean to study the effect of DNA demethylation in cellular differentiation in growth plate and articular chondrocytes in horses. Chondrocytes were isolated from growth plate and articular cartilage of few days old foals, treated or not with 5-aza for 4 days then the cells were divided into 3 groups: control (did not receive 5-aza), 5-aza cont (continued to receive 5-aza), 5-aza rec (previously treated with 5-aza). These were treated or not with BMP-2 or thyroid hormone, agents known to induce chondrocyte differentiation in susceptible chondrocytes. The cells were then collected at days 4, 8 and 12 and gene expression of the chondrocytic markers collagen type II and aggrecan and the chondrocytic differentiation markers was analyzed by Northern Blot analyses and quantitative PCR. The results showed that 5-aza variably increase the responsiveness to growth plate chondrocytes to BMP-2, however is not able to induce hypertrophic transformation in articular chondrocytes.

In conclusion, our results suggest that DNA methylation play a role in chondrocyte differentiation and articular cartilage homeostasis. Further studies using high throughput technology are warranted to characterize the methylation profile of normal articular cartilage, aged cartilage and osteoarthritic cartilage to further understand how DNA methylation plays a role during cellular differentiation and disease progression.

To family and friends

## ACKNOWLEDGEMENTS

Thank you to The Arthritis Foundation, Beckman Institute, Illinois Department of Human Services and the Department of Veterinary Biosciences for financial support. This project would have not been possible without the participation and support of many people. Many thanks to my advisor, Dr. Matthew Stewart, for being patient and supportive. Thank you for reviewing and helping to make some sense of the data that seemed to make no sense. Thanks to Dr. Allison Stewart and Dr. Richard Evans for input given during the beginning of this project. Also, many thanks to Dr. David Bunick, Dr. Pamela Wilkins, Dr. Sherrie Clark and Dr. Peter Jones for sharing your knowledge and helping to conclude this project. Special thanks to Dr Peter Jones for believing in me and giving encouragement when I thought that “model organisms & epigenetics” was way over my head. Many thanks to all the students who passed through the Stewart’s lab for bringing a new prospective and reminding me about why I got into this program to begin with (teaching). Thank you Yimin, Trina and Holly, for passing along all your experience in the lab as well as for the friendship. Thank you my lab mates Jill, Little Matt, Yuwen and Sushmitha for all the help during all stages of this project and for the friendship as well. Many thanks to my sisters of heart, Elena Silva and Fernanda Cunha, for being my foundation and support in the final stages of this journey. Finally thanks to my lovely family for giving me the strength to continue on this journey.

“Tenho pensamentos que, se pudesse revelá-los e fazê-los viver, acrescentariam nova luminosidade às estrelas, nova beleza ao mundo e maior amor ao coração dos homens...” Fernando Pessoa

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## **CHAPTER 1: INTRODUCTION**

There is increasing amount of data indicating that epigenetics play a role in aging and aging-related diseases. Of the age-related disease, osteoarthritis is of particular importance to current health issues. Osteoarthritis (OA) is a progressive, degenerative disease that affects articular cartilage, the synovial lining and adjacent bone of joints. OA currently affects over 30% of the US population. This figure is almost certain to increase as the American population ages and the childhood obesity “epidemic” impacts the consequent development of affected individuals. OA is also an extremely common presenting complaint in veterinary patients such as performance horses and several popular dog breeds.

Despite the considerable societal burden associated with OA, current medical and surgical therapies for this disease are largely palliative, targeted at controlling the severity of clinical signs without affecting disease progression. Consequently, total joint replacement is the current “gold standard” for the treatment of OA; a highly invasive and costly surgical procedure with its own attendant morbidities and complications. Recently, the results of several published studies have linked changes in the chondrocytic phenotype that occur during OA to the epigenetic status of chondrocytes. Previous research done in the Stewart lab suggests that DNA methylation is involved in the regulation of the chondrocytic phenotype. Therefore, the research presented in this dissertation is focused on characterizing the effects of DNA methylation on the chondrocytic phenotype, identifying the mechanisms by which DNA demethylating agents alter the chondrocytic phenotype and defining the role of epigenetics in chondrocyte phenotypic stability, control of differentiation and the pathogenesis of OA.

The long-term objective of this research program is to develop strategies using epigenetic modifying-agents for the prevention or mitigation of OA progression.

Briefly, this thesis is composed by a literature review and three data-based chapters, presented as manuscripts. The literature review covers the topics of osteoarthritis and the different aspects that contribute to disease initiation and perpetuation, as well introducing the concepts of epigenetics and what it is currently known regarding epigenetic influences on chondrocytes and on osteoarthritis.

The first study (Chapter 3) addresses the phenotypic effects of 5-azacytidine (5-aza) in human articular chondrocytes and investigates whether upregulation of collagen type II, observed after 5-aza treatment, is associated with demethylation of the promoter and enhancer CpG island. In this chapter, we also mapped the methylation status of the collagen type II CpG island in cartilage, bone, and several chondrogenic and non-chondrogenic cell lines.

The second study (Chapter 4) was prompted by the study of Zimmermann and colleagues, published in 2008. This study correlated collagen type X expression during stem cell chondrogenesis with demethylation of two specific CpGs in the collagen type X promoter. We analyzed whether similar demethylation events occurred in articular chondrocytes during osteoarthritis.

The third study (Chapter 5) investigated the phenotypic effects of 5-aza on equine articular and growth plate chondrocytes. More specifically, we studied whether 5-aza administration directly stimulated expression of hypertrophic genes in articular and growth plate chondrocytes, and whether 5-aza administration affected the phenotypic responses of articular and growth plate to hypertrophic stimulation with BMP-2.



## **CHAPTER 2: LITERATURE REVIEW**

### **GENESIS AND PHENOTYPIC CHARACTERISTICS OF CHONDROCYTE POPULATIONS**

#### **Chondrogenic differentiation of MSCs and chondrocytic phenotype**

During embryonic skeletogenesis, chondrogenesis is initiated by condensation of mesenchymal stem cells (MSCs) derived from somitic or lateral plate mesoderm <sup>1</sup>. Signals from several morphogens (Transforming growth factor [TGF] - $\beta$ ; Fibroblast growth factors [FGF] -2,-4,-8,-10; Bone morphogenetic protein [BMP] -2, -4, -7; Sonic Hedgehog [Shh] and Wnts 3A and 7A), mediated by transcription factors Hox A, Hox D, Sox-9, Smads and Gli-3, act in a temporally and spatially regulated manner to drive proliferation and condensation of MSCs. During this process, the cells undergoing chondrogenesis are characterized by expression of the following extracellular matrix proteins: fibronectin, N-cadherin, tenascin, cartilage oligomeric protein (COMP) and collagen types I, IIA and XI. The chondroprogenitor cells proliferate and differentiate to form a cartilage template, or anlagen, then undergo endochondral ossification to form the axial and appendicular skeletal elements. Chondro-progenitor proliferation is driven by insulin growth factor (IGF) -1, FGF-2, BMPs -2, -4, -7, and -14 <sup>2</sup>. In this phase, the transcription factors Sox-9, L-Sox-5 and Sox-6 play key roles, controlling expression of several chondrocyte-specific genes such as collagen type IIB (Col II), aggrecan (Agg), collagen type XI (Col XI) and link protein (LP) <sup>3-11</sup>. Collectively, signaling pathways mediated by the secreted ligands parathyroid hormone-related peptide (PTHrP), FGFs,

Indian hedgehog (Ihh), TGF- $\beta$ s, BMPs, and Wnts interact to control the rate of chondrocyte proliferation and hypertrophic differentiation<sup>2, 12-14</sup>.

The hypertrophic stage of differentiation is characterized by expression of collagen type X (Col X) and alkaline phosphatase (ALP). Hypertrophic chondrocytes also express several matrix metalloproteases (MMPs 2, 3, 9 and 13) and vascular endothelial growth factor (VEGF)<sup>15, 16</sup>. These factors remodel the hypertrophic cartilage matrix and stimulate endothelial progenitor migration, in preparation for vascularization. Terminally, hypertrophic chondrocytes undergo apoptosis, and the acellular cartilage matrix is colonized by osteoblast precursors that invade the matrix along with vascular endothelial elements<sup>12, 14</sup>.

### **Phenotypic similarities and differences between cartilage tissues**

Cartilage is a connective tissue characterized by a small number of cells (chondrocytes) interspersed by large amounts of extracellular matrix. Chondrocytes engaged in the process of endochondral ossification generate cartilage that forms temporary anlagen during skeletal development. Endochondral cartilage persists after skeletal development in the growth plates and secondary centers of ossification of growing bones and in fracture callus<sup>1, 17, 18</sup>. Endochondral cartilage is ultimately replaced by bone, although the growth plate cartilages of actively growing long bones persist until puberty. In contrast to the transient cartilage at endochondral locations, permanent cartilage persists throughout life. Permanent cartilage is found in several anatomical locations and is categorized into three types depending on extracellular matrix composition. In all locations, cartilage provides solid but deformable structural support.

Elastic cartilage is rich in elastin and is found in the ear and epiglottis. Fibrocartilage contains collagen type I and it is located in intervertebral discs, menisci, compressive regions of tendons and the pubic symphysis. Hyaline cartilage is rich in collagen type II and proteoglycans and it is found in the ribs, larynx, trachea, and bronchi, nose and, of particular clinical importance, on the articular surfaces of joints.

Articular cartilage is a highly specialized connective tissue that covers the articulating surfaces of bones. It is avascular and rich in extracellular matrix composed predominantly of water, collagen type II and proteoglycans. The collagen fibrils provide the cartilage with tensile strength and resistance to shear, while aggrecan provides resistance to compressive loads <sup>19,20</sup>. Articular chondrocytes do not undergo hypertrophic maturation under normal circumstances. Instead, articular chondrocytes persist throughout life in a nominally pre-hypertrophic state, characterized by the expression of Col II and Agg, with little or no Col X expression or ALP activity <sup>21</sup>.

### **Maintenance of the chondrocytic phenotype in articular cartilage**

The mechanism by which articular chondrocytes avoid hypertrophy is still not completely understood. Several studies indicate that TGF- $\beta$  signaling is mandatory for joint formation and maintenance of articular cartilage. TGF- $\beta$  receptor II (Tgfr2) is highly expressed in developing joints and lack of Tgfr2 impairs interphalangeal joint development <sup>22</sup>. Further, loss of TGF- $\beta$  signaling results in decreased cartilage synthesis and accelerated degradation of proteoglycans, resulting in articular cartilage changes that resemble osteoarthritis <sup>23-25</sup>.

BMPs, also members of the TGF- $\beta$  superfamily, also play a role in cartilage formation and chondrocyte phenotypic maintenance<sup>1</sup>. While single BMP receptor knockout mutants (BMP receptor 1A or BMP receptor 1B) form cartilage normally, double mutant mice are chondrodysplastic. This indicates that BMP signaling is needed for proper chondrogenesis, but some functional receptor redundancy exists in BMP signaling<sup>26</sup>. BMP signaling is also important for cartilage maintenance, since reduced signaling activity leads to cartilage degeneration<sup>27</sup>. *In vitro*, articular chondrocytes cultured as monolayers down-regulate endogenous expression of BMP-2 concurrent with the loss of the chondrocytic markers Col II and Agg. Addition of exogenous BMP-2 slows the dedifferentiation process and sustains Col II and Agg expression. Moreover, Noggin and a soluble BMP receptor construct, both BMP antagonists, reduced Col II and aggrecan expression in aggregate cultures; a culture condition known to maintain the chondrocytic phenotype<sup>28</sup>. Taken together, these findings confirm that BMP activity is also necessary for maintenance of the articular phenotype<sup>29</sup>.

PTHrP is a secreted ligand that plays a role in maintaining chondrocytes in a pre-hypertrophic state. Weir and others overexpressed PTHrP using a Col II promoter in a transgenic model and caused strong inhibition of chondrocyte terminal differentiation in growth plates<sup>30</sup>. Also, PTHrP-deficient and PTHrP receptor-deficient mice exhibit a reduced proliferative stage of differentiation and a concurrent accelerated entry into the hypertrophic phase of differentiation<sup>31, 32</sup>. During endochondral bone formation, PTHrP mediates the effects of Ihh on chondrocyte maturation through a negative feedback loop. Ihh, secreted by chondrocytes at the transitional boundary between the pre-hypertrophic and hypertrophic zones, stimulates the synthesis of PTHrP by periarticular and

perichondrial cells at the nascent articular surface. PTHrP diffuses to act on pre-hypertrophic chondrocytes which express the PTH/PTHrP receptor, to slow the rate of differentiation<sup>33, 34</sup>. There is evidence that PTHrP's effects on hypertrophic inhibition are partially mediated by inhibition of RUNX-2 activity, a transcription factor mandatorily required for chondrocyte hypertrophy<sup>35, 36</sup>.

The role of PTHrP in the maintenance of articular chondrocytes has recently been clarified by the Broadus laboratory. This group developed a PTHrP-lacZ knock-in murine model which increases the sensitivity of local PTHrP detection by approximately five-fold. In these mice, PTHrP expression was detected in the proximal growth plate and superficial layer of articular cartilage during development. The articular expression profile persists after skeletal maturity is reached. They also demonstrated that PTHrP expression is load-induced in articular chondrocytes. Mechanical unloading of joints decreased the expression of PTHrP and was associated with articular chondrocyte hypertrophy<sup>37-39</sup>. Given that PTHrP expression was restricted to the superficial zone of articular cartilage and this zone is the earliest and most severely affected subpopulation of chondrocytes in arthritis, it is highly likely that aberrant PTHrP secretion contributes to osteoarthritic progression. However, the extent to which the chondrocyte phenotypic changes that occur in osteoarthritis result from decreased PTHrP expression needs to be determined.

## **PATHO-PHENOTYPIC ASPECTS OF OSTEOARTHRITIS**

### **Pathogenesis of osteoarthritis**

Osteoarthritis (OA) is the most prevalent age-related disease in the world. There are no methods for curing the disease once cartilage degeneration is established and, with the exception of total joint arthroplasty, the currently available non-surgical treatments are largely palliative. OA affects over 60% of the US population over 65 years of age <sup>40</sup> and OA-related costs total more than \$60 billion per year in the United States economy alone <sup>41, 42</sup>.

OA is a non-inflammatory disease characterized by progressive articular cartilage degradation <sup>43</sup>, calcification <sup>44</sup>, and subchondral bone alterations <sup>45</sup>. The disease is notable for the relative absence of neutrophil and macrophage infiltration, but high levels of inflammatory cytokines are produced by synoviocytes and chondrocytes <sup>43, 46, 47</sup>, resulting in “inflammation-like” cellular responses. At the cellular level, OA is characterized by two phases: an initial biosynthetic phase, during which the chondrocytes increase matrix synthesis in efforts to repair damaged cartilage; and a secondary degradative phase, in which the enzymes produced by chondrocytes and synoviocytes degrade the cartilage matrix and overwhelm any new matrix synthesis <sup>48</sup>. Moreover, cartilage degradation products derived from the cleavage of fibronectin, collagen type II and matrilins also stimulate degradative enzyme secretion, generating a progressive cycle of cartilage destruction <sup>49-52</sup>. As an example, fibronectin fragment levels are increased in osteoarthritic synovial fluid <sup>53</sup> and these fragments stimulate metalloproteinase release by bovine articular cartilage explants <sup>49</sup>.

There are two main theories regarding osteoarthritis initiation and progression. The first theory emphasizes the consequences of repetitive mechanical injury to articular cartilage, while the second theory focuses on chondrocyte senescence<sup>43, 54</sup>. The “repetitive mechanical injury” model is corroborated by observations that cartilage matrix stiffness increases in OA, due to increased collagen cross-linking, with a concomitant decrease in compressive resistance due to decreased aggrecan monomer size<sup>55-58</sup>. The second theory is based on a model where focal proliferation, cumulative oxidative stress and mitochondrial dysfunction impair chondrocytes’ capacity to maintain the extracellular matrix<sup>54, 59</sup>. The role of oxidative stress in osteoarthritis was corroborated by large-scale DNA array analyses of human articular cartilage. Seventy-eight human cartilage samples were screened using an oligo array covering over 4,000 genes. A significant downregulation of anti-oxidative genes such as superoxide dismutases 2 and 3 and glutathione peroxidase 3 was observed in OA samples<sup>60</sup>.

Besides repetitive mechanical injury and chondrocyte senescence, obesity and age-related alterations in the content or consequences of growth factor signaling also appear to contribute to the pathogenesis of OA<sup>40, 41, 43, 58, 61</sup>. Collectively, the factors driving progression of OA lead to a persistent imbalance between the biosynthetic and degradative activities in articular cartilage, resulting in irreversible degeneration.

### **Role of inflammatory cytokines in OA**

Several inflammatory cytokines are up-regulated in OA. These cytokines stimulate chondrocytes and synoviocytes to secrete degradative enzymes such as MMPs and ADAMTSs (a disintegrin and metalloprotease with thrombospondin motifs)<sup>62-65</sup>.

MMPs predominantly degrade collagens while ADAMTSs cleave aggrecan. The importance of these enzymes in cartilage degradation has been demonstrated *in vitro*<sup>66-69</sup> and *in vivo*<sup>68, 70-73</sup>. Recent murine models strongly suggest that ADAMTS-5 is the major degradative enzyme responsible for cartilage degeneration in OA<sup>68, 74</sup>. However, given the very different cartilage matrix architecture and biomechanical loading stresses, the situation in humans and other higher mammals is likely to be more complex.

Besides inducing matrix degradative enzymes, cytokines also directly downregulate the synthesis of extracellular matrix proteins such as collagen type II<sup>75, 76</sup>, aggrecan and link protein<sup>41, 77</sup> by articular chondrocytes. Interleukin (IL)-1 $\beta$ , a major inflammatory cytokine in OA, down-regulates collagen type II expression by articular chondrocytes by suppressing Sox-9. Sox-9 down-regulation was linked to IL-1 $\beta$ -induced expression and phosphorylation of c-Jun, with consequent activation of the AP-1 transcription factor complex<sup>75</sup>.

IL-6 down-regulates the expression of several cartilage-specific matrix genes, such as collagen type II, aggrecan and link protein, by articular chondrocytes<sup>76-78</sup>. Downregulation of collagen type II is linked to an increase in trans-inhibitory Sp3 occupancy in the promoter region of the Col II gene<sup>76, 78</sup>.

### **Growth factor activities and dysfunction in OA**

TGF- $\beta$  signaling is not only significant for maintenance of healthy articular cartilage, but also has chondroprotective activities in OA, by counteracting the effects of inflammatory cytokines<sup>67, 79-81</sup>. TGF- $\beta$ s stimulate expression of tissue inhibitors of metalloproteases (TIMPs), and increase collagen type II and proteoglycan synthesis<sup>82-85</sup>



by osteoarthritic chondrocytes. TGF- $\beta$ 2 inhibits the expression of MMP-13 and MMP-9 by human cartilage explants and consequently decreases collagen type II degradation<sup>81</sup>. This collagen-protective effect can be attributed to Smad-dependent upregulation of TIMP-3, an inhibitor of the major cartilage degradative enzymes MMP-13 and ADAMTS-4<sup>84</sup>. TIMP upregulation by TGF- $\beta$ 1 is also effective in the presence of the inflammatory cytokine tumor necrosis factor (TNF)- $\alpha$ <sup>67</sup>.

Considering the potential therapeutic benefits of TGF- $\beta$  activity in OA, clinical applications of these activities have not been successful, largely due to the diverse range of effects of TGF- $\beta$  on articular and periarticular tissues. Multiple TGF- $\beta$  injections in the femorotibial joints of mice induced cartilage destruction and osteophyte formation<sup>86</sup>. This deleterious effect was attributed to TGF- $\beta$ -induced upregulation of MMP-13, one of the major collagenases implicated in osteoarthritis<sup>87</sup>. To clarify the role of TGF- $\beta$  in OA, Takahashi and co-workers<sup>80</sup> used genome-wide expression arrays to assess the pathways affected by TGF- $\beta$  and IL-1 treatment in the H4 murine chondrocytic cell line. In this experiment, cluster analyses confirmed the protective role of TGF- $\beta$  by selective counteraction of inflammatory/catabolic IL-1-induced genes, but the analyses also identified a cluster of genes that may be responsible for the adverse effects induced by TGF- $\beta$ , such as Connective tissue growth factor (CTGF) and VEGF. These factors are known to induce cartilage angiogenesis and stimulate MMP production<sup>88,89</sup>, exacerbating osteoarthritic pathology.

Just as BMPs are critical for normal articular cartilage development and maintenance, several BMP ligands have demonstrated chondro-protective effects in the context of OA. Osteogenic protein-1 (OP-1), also referred to as BMP-7, confers a

protective effect against cartilage degradation in osteoarthritis. OP-1 increases aggrecan and collagen type II protein secretion by human articular chondrocytes<sup>90</sup> and restores proteoglycan secretion to control levels by articular chondrocytes challenged with IL-1<sup>91</sup> or fibronectin fragments<sup>92</sup>. OP-1 protective effects were also demonstrated in a rabbit model of OA. Joints treated with OP-1 showed less surface fibrillation and cartilage deterioration compared to the control group, had significantly greater expression of aggrecan and collagen type II, and less aggrecanase expression<sup>93</sup>. Immortalized human chondrocyte C-28I2 cells and human primary chondrocytes challenged with IL-1 or fibronectin fragments, decreased MMP-13 mRNA and protein expression when treated with OP-1 or IGF-1<sup>94</sup>.

Bmp-2 is also chondro-protective by stimulating extracellular matrix synthesis by chondrocytes *in vivo* and *in vitro*<sup>29, 95-97</sup>. This is corroborated by the fact that mice lacking BMP receptor signaling develop cartilage degeneration similar to OA<sup>27</sup>. BMP-2 has shown to modulate collagen type II expression, favoring ‘mature’ collagen type II B production over the type II A ‘immature’ variant, by normal and osteoarthritic chondrocytes<sup>98</sup>. However, BMP-2 also plays a role in chondrocyte hypertrophy and osteophyte formation in OA<sup>95, 99, 100</sup>. It has been suggested that an imbalance between TGF- $\beta$  and BMP signaling pathways leading to stimulation of Smad1/5 responsive genes directs chondrocyte to hypertrophy in OA<sup>21, 101</sup>.

IGF-1 also has chondro-anabolic activities<sup>102, 103</sup>. IGF-1 increases glycosaminoglycan content and synthesis and collagen type II expression by equine chondrocytes suspended in fibrin<sup>104</sup>. *In vivo*, a composite of chondrocytes and polymerized fibrin induced better healing of large full-thickness cartilage defects in

horses when IGF-1 was added <sup>105</sup>. In addition to increasing the anabolic activity of chondrocytes, IGF-1 counteracts the degradative effects caused by cytokine administration. TNF- $\alpha$ , IL-1 and oncostatin M stimulated the expression of cartilage degradative enzymes MMP-1, -3, -8 and -13, by human and bovine chondrocytes <sup>67, 106</sup>. IGF-1 treatment reduced cytokine-induced collagen degradation and antagonized MMP-1, -3, and -8 upregulation by these cytokines <sup>67, 106</sup>.

There is some evidence suggesting that aging plays a role in the dysregulation of growth factor signaling pathway and responses in chondrocytes, leading to cartilage degradation and phenotypic alterations <sup>25, 85, 107</sup>. As examples, TGF- $\beta$ 2, TGF- $\beta$ 3 and TGF- $\beta$  receptor expression is decreased in old mice <sup>25</sup>. Corroborating this finding, TGF- $\beta$  signaling deficiency, through targeted disruption of Smad3 <sup>24</sup> or overexpression of a truncated, non-functional Tgfr2 <sup>23</sup> leads to articular cartilage changes that resemble osteoarthritis.

Recent evidence indicates that age-related changes in TGF- $\beta$ /BMP receptor expression could contribute to OA initiation and progression <sup>108</sup>. TGF- $\beta$ s conventionally signal through Smads 2 and 3, but these ligands are also able to signal through the activin receptor-like kinase (ALK) -1 receptor in the absence of more high affinity receptors, activating the Smad1/5/8 pathway, which leads to hypertrophic differentiation <sup>85</sup>. There is an 80% decrease in ALK5 expression in the articular cartilage of aged mice, while ALK1 expression is relatively unaffected (17% reduction). Overexpression of ALK1 led to phosphorylation of Smad1/5/8 and upregulation of MMP-13; an enzyme secreted by hypertrophying growth plate chondrocytes. In addition, downregulation of ALK5 by small hairpin (sh) RNA induced MMP-13 expression while ALK1 shRNA abolished

MMP-13 expression. Taken together, these data indicate that an increase in ALK1/ALK5 ratio leads to chondrocyte hypertrophy, activation of MMP-13, and collagen type II degradation in OA<sup>108</sup>.

### **Biomechanical influences**

Given that cartilage is a hypocellular tissue (3-5% cellularity) rich in extracellular matrix and subject to continuous biomechanical loading, the maintenance of tissue architecture and function is heavily dependent on chondrocyte interactions with the extracellular matrix (ECM), through cell membrane receptors. These receptors include integrins that bind fibronectin and collagens, the discoidin domain receptors (DDR) that bind to collagens; CD44, CD62 and intercellular adhesion molecule-1 (ICAM-1) that bind hyaluronan<sup>109, 110</sup>. Integrins activate major signaling pathways and promote cell adhesion, migration, differentiation, proliferation and cell survival<sup>50, 111</sup> and are essential for chondrogenesis<sup>112</sup>. These receptors also play a role in OA pathogenesis. Cartilage degradation products such as collagen, fibronectin and matrilin fragments, stimulate degradative enzyme secretion, propagating progressive cartilage destruction<sup>49, 51, 52</sup>.

Fibronectin-induced upregulation and activation of MMP-13 is activated by  $\alpha 1\beta 5$  integrin receptor, mediated by the MAP kinase signaling pathway<sup>113, 114</sup>.

DDRs are tyrosine kinase receptors that are activated through interaction with collagen<sup>110</sup>. More specifically, DDR-2 activation is dependent on interaction with the triple helix D2 period of collagen type II and influences adhesion, proliferation and extracellular matrix remodeling<sup>110, 115, 116</sup>. Mice with mutations of collagen type IX or XI exhibit upregulation of DDR-2 and MMP-13<sup>117, 118</sup> and develop osteoarthritis<sup>119, 120</sup>. *In*

*vitro* studies determined that MMP-13 upregulation was dependent on DDR-2 activation<sup>117, 118, 121</sup>. More recently, other groups demonstrated that DDR-2 expression correlated with MMP-13 and collagen type II degradation and OA grade in human osteoarthritis, using complementary immunohistochemical and quantitative PCR approaches<sup>121, 122</sup>. The consequent ECM degradation upregulates and activates DDR-2, propagating the cartilage destruction observed in OA<sup>121</sup>. In addition, DDR-2 also upregulates IL-6<sup>52</sup>, an inflammatory cytokine that induces MMP expression and inhibits proteoglycan and collagen type II deposition<sup>77</sup>.

CD44 is a transmembrane protein that interacts with cytoskeletal proteins allowing for changes in the extracellular matrix environment to be transduced intracellularly. It functions as the main hyaluronan receptor and is involved in cellular adhesion, migration and differentiation<sup>123, 124</sup>. In chondrocytes, CD44 is responsible for attachment of hyaluronan-proteoglycan aggregates to chondrocytes' surface and needed for maintenance of cartilage homeostasis<sup>125-127</sup>. The importance of CD44 expression and extracellular homeostasis was demonstrated by siRNA inhibition. Chondrocytes and cartilage treated with CD44 antisense oligonucleotides showed depletion of extracellular proteoglycan similar to osteoarthritis<sup>127</sup>. Moreover, in chondrocytes, CD44 has been shown to be involved in internalization and turnover of hyaluronan and G1 aggrecan domain<sup>128, 129</sup> and signal transduction<sup>130, 131</sup>. Therefore, the ability to repair cartilage damage is not only dependent on upregulation of matrix extracellular genes but also in the capability to incorporate those proteins to the extracellular matrix to re-establish the pericellular environment<sup>125, 126</sup>. In osteoarthritis, the massive accumulation of hyaluronan and G1 aggrecan fragments due to extracellular matrix degradation may overcome CD44

turnovers capability. This may lead to lack of binding sites to newly formed hyaluronan and proteoglycans impeding cartilage repair <sup>132</sup>.

### **Subchondral bone alteration and cartilage calcification**

Subchondral bone alteration is a major pathological characteristic of arthritis. The extent to which these alterations influence osteoarthritic progression is not well defined. Clearly, subchondral bone sclerosis will alter the biomechanical loading experienced by the adjacent articular cartilage. However, there is also evidence supporting direct effects of subchondral osteoblasts on articular chondrocyte activities. Human chondrocytes suspended in alginate beads were co-cultured with osteoblast monolayer cultures, isolated from sclerotic or non-sclerotic bone. Sclerotic osteoblasts inhibited aggrecan gene expression and protein secretion and also increased MMP-3 and -13 gene expression by chondrocytes. Similar results were obtained when non-sclerotic osteoblasts were pre-incubated with inflammatory cytokines (IL-1 $\beta$ , IL-6 or oncostatin M (OSM)) prior to co-culture <sup>133</sup>. In another study, sclerotic osteoblasts decreased expression of collagen type II, Sox-9, PTHrP and its receptor (PTH-R) and increased expression of osteoblast stimulating factor 1 (OSF-1) by articular chondrocytes <sup>45</sup>. Taken together, signals from osteoblasts in the sclerotic subchondral bone compartment could contribute to cartilage degradation and phenotypic shift of articular chondrocytes in OA.

### **Patho-phenotypic characteristics of OA chondrocytes**

Although OA is predominantly characterized by loss of ECM homeostasis, the disease also causes distinct phenotypic alterations in the resident chondrocyte population.

These phenotypic changes resemble the transitions that occur during hypertrophic differentiation of endochondral lineage chondrocytes<sup>134</sup>. Articular chondrocytes down-regulate Col II and Agg expression<sup>48, 135</sup> and begin to express Col X<sup>21, 136-139</sup>. This has been demonstrated by microarray comparisons<sup>140</sup> and differential Col X immunostaining in OA and normal cartilage<sup>138, 141</sup>. Aberrant expression of other hypertrophic markers such as ALP and osteocalcin (OCN) have also been linked to OA<sup>141, 142</sup>. Osteocalcin mRNA and protein expression were increased in the subchondral bone plate from patients with severe OA and was also present in OA chondrocytes in the deep cartilage layer and in chondrocyte clusters<sup>141</sup>. Late stage OA samples also were positive for Col X and ALP expression, which reinforces the hypertrophic-like phenotypic change that occurs in osteoarthritis chondrocytes.

Articular chondrocytes also upregulate expression of MMPs that are physiologically expressed, by hypertrophic chondrocytes, during the final stage of hypertrophic differentiation<sup>15, 16</sup>. In endochondral cartilage, MMPs act to remodel hypertrophic cartilage in preparation for vascularization and matrix mineralization. In articular cartilage, increased MMP activity results in cleavage of critical ECM proteins and consequent tissue compromise. Therefore, dysregulation of mechanisms involved in chondrocyte differentiation and phenotypic stability are directly implicated in the pathogenesis of OA.

Mineralization of cartilage is a recognized pathological alteration in OA. Fuerst and co-workers analyzed the excised cartilage specimens from humans undergoing total joint replacement. All one hundred and twenty specimens showed mineralization of cartilage that correlated with Col X expression and chondrocyte hypertrophy. The

secreted minerals were identified as basic calcium phosphates; the same crystals known to be produced by hypertrophic chondrocytes during endochondral ossification<sup>44</sup>. The close relationship between ALP activity, matrix vesicle formation and cartilage mineralization in osteoarthritic cartilage, was demonstrated using electron microscopy<sup>142</sup>. The increase in number of apoptotic cells in osteoarthritic cartilage has also been linked to cartilage calcification<sup>143</sup>.

The mechanisms that drive articular chondrocyte phenotypic destabilization in OA are largely unknown. Age-related changes in TGF- $\beta$  and BMP signaling pathways and activities are obvious candidates that could contribute to osteoarthritis pathogenesis<sup>40, 41, 43, 58, 61, 108</sup>. In addition, the recent findings that PTHrP are expressed by murine articular cartilage subpopulations after skeletal maturity was reached<sup>37, 39</sup>, suggest that loss of PTHrP expression by articular chondrocytes, is a key event facilitating chondrocyte hypertrophy during OA.

Ihh is a secreted ligand involved in skeletogenesis and chondrocyte differentiation. Since Ihh is linked to PTHrP activities in endochondral chondrocytes, the participation of Ihh in the pathogenesis of OA has been addressed. Lin and co-workers used several transgenic mouse models to demonstrate that activation of hedgehog signaling leads to upregulation of genes known to be increased in OA such as ADAMTS-5, COL10A1, RUNX-2 and MMP-13, along with radiographic and histologic changes characteristic of OA<sup>144</sup>. Accepting these findings, the mechanism(s) by which Ihh could be upregulated in OA cartilage has not yet been identified.



## **EPIGENETIC INFLUENCES ON CELL DIFFERENTIATION AND PHENOTYPE**

Epigenetics refers to “mechanisms that initiate and maintain patterns of gene expression and gene function in a heritable manner without changing the sequence of the genome”<sup>145</sup>. Epigenetics impact gene regulation through DNA methylation, histone protein modifications and chromatin organization. Overall, DNA methylation of CpG islands is associated with histone hypoacetylation that collectively repress transcription. DNA methyltransferases methylate cytidines at CpG dinucleotide sites. This modification can interfere with the recognition and attachment of DNA binding proteins to genomic *cis* regulatory elements. Additionally, methyl-CpG binding proteins are recruited to methylated CpGs (mCpG) and, in turn, recruit histone deacetylases (HDACs), histone methyltransferases (HMTs) and chromatin-remodeling factors to methylated regions. These modifications condense chromatin structure, sequestering potential binding sites from transcriptional activators and restricting occupancy<sup>146</sup>. However, promoter occupancy does not depend solely in its epigenetic state. Transcription activation and repression are also regulated by competitive binding of promoter, enhancer and silencer elements<sup>76, 78, 147-150</sup>. Therefore, activation or repression of a gene is a collective consequence of the interplay of transcription factors and epigenetic constraints.

Epigenetic processes are involved in regulation of germline-specific genes, tissue-specific genes, imprinting, and aging<sup>151-155</sup>. A growing body of work indicates that epigenetics is also critical in a range of disease processes. For instance, the hallmark of

neoplastic cells is global DNA hypomethylation and hypermethylation of specific tumor suppressor genes which are transcriptionally suppressed<sup>156</sup>.

### **Histone modifications**

Histones are nuclear proteins involved in DNA packaging through chromatin formation. A nucleosome is a chromatin unit formed by a core of histones (two H2A-H2B dimers and a H3-H4 tetramer) and 147 base pairs of DNA wrapped around an octamer of histones. Modification of the histone amino-tails, in conjunction with other nuclear proteins, link histones and DNA methylation play a role regulating chromatin structure and gene accessibility<sup>157, 158</sup>.

Histone acetyltransferases (HATs) are responsible for the acetylation of specific lysine residues in the histone amino-tails. The GNAT, MYST, p300/CBP, SRC and TAFII250 families are known to be able to catalyze the transfer of acetyl residues to histone amino-tails, facilitating transcriptional activity<sup>159</sup>. On the other hand, histone hypoacetylation correlates with gene repression. Heterochromatin, for example, is characterized by histone (H) 3 lysine (K) 9 methylation, H4 hypoacetylation, CpG methylation and presence of the heterochromatin protein HP1. Methylated H3K9 and the DNA methyltransferases (Dnmts) serve as binding sites for heterochromatin (HP) -1 and methyl binding proteins (MBPs)<sup>160</sup>. MBPs recruit histone deacetylases (HDACs) and chromatin-remodeling proteins, leading to deacetylation of histone amino-tails, chromatin compaction and repression of gene expression<sup>161, 162</sup>. While methylated H3K9 is located mainly in pericentromeric heterochromatin, H3K27 trimethylation correlates with silencing of homeotic genes and also is found in the inactive X chromosome<sup>163</sup>. H3K27

trimethylation is carried out by the Polycomb methyltransferase EZH2<sup>164</sup> that has been shown to associate with Dnmts, and to be necessary to establish DNA methylation and silencing of genes involved in development. On the other hand, activation of transcription of genes involved in development is mediated by the Trithorax complex which is associated with H3K4 trimethylation<sup>165-168</sup>.

Genome-wide screening has also defined a range of histone marks that correlate with functional activities of genomic sequences such as transcript-coding sequences<sup>223</sup>, exon boundaries<sup>227</sup>, active enhancers<sup>221</sup>, telomeric and long-terminal repeat sequences<sup>223</sup>. It is apparent that histone modifications, in conjunction with the transcriptional machinery comprised of RNA polymerase and associated transcription factors, collectively delineate the transcriptional state of the genome.

## **DNA methylation**

DNA methylation is a post-replication modification catalyzed by DNA methyltransferases. In mammals, the Dnmts catalyze the transfer of a methyl group from S-adenosyl-methionine to the carbon 5 of cytosine in the CpG dinucleotides. Cytosine methylation in CHG and CHH sites (where H= A, T or C) is found in embryonic stem cells and disappear after differentiation<sup>169</sup>. Other forms of DNA methylation found in humans are methylcytosines and hydroxymethylcytosines<sup>170</sup>, but their significance still remains unknown. To date, five Dnmts have been identified: Dnmt1, Dnmt2, Dnmt3A, Dnmt3B and Dnmt3L. However, only Dnmt1, 3A and 3B have methyltransferase activity *in vivo*. Dnmt2 has very little methyltransferase activity *in vitro*<sup>171</sup>. More recently, its methyltransferase activity was reported to regulate retrotransposon silencing and

telomere integrity in *Drosophila melanogaster*<sup>172</sup> and to methylate tRNA<sup>173</sup>. Dnmt3L lacks the methyltransferase catalytic domain but contributes to *de novo* methylation by interacting with the catalytic domains of Dnmt3A and Dnmt3B, enhancing their enzymatic activity<sup>174, 175</sup>. Considering that Dnmts1, 3A and 3B may have some overlapping function<sup>176</sup>, Dnmt1 primarily functions to maintain the genomic methylation status following replication. By interacting with proliferating cell nuclear antigen (PCNA), Dnmt1 detects hemi-methylated sites during replication and methylates the newly replicated DNA strand<sup>177</sup>. In contrast, Dnmt3A and 3B are primarily responsible for *de novo* DNA methylation<sup>178</sup>. All these methyltransferases are critical for embryogenesis, since deletions of any DNA methyltransferase (Dnmt1, Dnmt3A or Dnmt3B) result in embryonic lethal phenotypes<sup>179</sup>.

CpG dinucleotides present a bimodal distribution in the genome, reflecting an evolutionary feature. Most of the genome is CpG-poor due to cumulative deamination of methylated cytidines to thymidines, which generates a TpG mutation after replication. However, deamination of non-methylated cytidines forms uridines which are easily detected by the excision repair system and therefore persist as CpGs in the genome<sup>171</sup>. There are specific areas in the genome that hold a greater concentration of CpG dinucleotides than expected, based on 'background' incidence. These regions are termed 'CpG islands' and correlate with promoter sequences of housekeeping genes and approximately 40% of tissue-specific genes<sup>180, 181</sup>. Takai and Jones (2002) defined CpG islands as regions longer than 500bp, containing more than 55% CGs and an observed CpG/expected CpG ratio of 0.65<sup>182</sup>. CpG islands localized in promoter regions are

usually unmethylated in normal tissues, regardless of the transcriptional activity of the gene<sup>183</sup>.

DNA methylation can repress transcription through transcription factor binding exclusion<sup>184, 185</sup>. As an example, CpG methylation is known to interfere with CTCF binding and regulate expression of H19/Igf2 imprinted genes. CTCF is an insulator that binds DNA when its consensus sequence is unmethylated. In such cases, CTCF binds to a region between the gene promoter and enhancer, silencing the maternal copy of the Igf2 gene. On the other hand, when the CTCF binding site is methylated, CTCF is unable to bind and the paternal copy of the Igf2 gene is expressed<sup>186, 187</sup>.

Methylated CpGs are also recognized by proteins that have a methyl-binding domain (MBD). To date, several methyl-binding proteins have been identified: MeCP2<sup>188</sup>, MBPs 1-4 and Kaiso<sup>189</sup>. Recently, Ho and colleagues demonstrated that MeCP2 contacts the water molecule tightly bound to the major groove of methylated DNA rather than methylated cytosine itself<sup>190</sup>. Methyl-binding proteins interact with histone deacetylases, histone methyltransferases (HMTs) and chromatin-remodeling complexes, resulting in chromatin compaction and gene silencing<sup>161</sup>.

Several epigenetic marks have been linked to gene silencing through the formation of heterochromatin. However, the sequence of mark acquisition and the relative importance of specific genomic alterations are somewhat controversial. The importance of CpG methylation is emphasized by the observation that methylated transgenes bind methyl-CpG-binding proteins that recruit HDACs and result in silencing<sup>191-193</sup>. Fuks and co-workers showed that MeCP2 associates with histone methyltransferases at H3K9, linking DNA methylation to consequent chromatin modifications that reinforce gene

silencing<sup>194</sup>. In contrast, findings in *Neurospora crassa* and *Arabidopsis thaliana*, suggest that DNA methylation is a secondary event that follows gene silencing by histone modifications. For instance, the histone methyltransferase, *dim-5*, is necessary for DNA methylation in *N. crassa*<sup>195</sup>. Similarly, when kryptonite, a HMT, is mutated in *A. thaliana*, major loss of DNA methylation occurs, leading to reactivation of transcription of several normally silenced genes<sup>196, 197</sup>. In mammals, DNMT1 and 3A were shown to be associated with SUV39H1 (a HMT that methylates H3K9)<sup>160</sup>. This suggests that DNMTs could be recruited to sites marked by H3K9 methylation through interaction with SUV39H1, and that histone methylation would therefore be required for DNA methylation. On the other hand, Mustkov and co-workers showed that a transgenic cassette stably integrated in chicken erythroid cells was inactivated primarily by hypoacetylation of H3 and H4 and loss of methylation at H3 lysine 4<sup>198</sup>. In this system, histone H3 K9 and promoter DNA methylation were secondary events and histone and DNA methylation occurred at the same time<sup>198</sup>. Taken together, the current data indicate that gene silencing involves coordinated and complementary DNA modification and protein complex interactions to achieve transient or permanent gene silencing.

## **MicroRNAs**

MicroRNAs (miRNAs) are ~ 22 nucleotide long, non-coding RNA molecules involved in posttranscriptional regulation of gene expression. They are transcribed by RNA polymerase II, processed in the nucleus by the RNase III Drosha and DGCR8 into precursor miRNAs, exported to the cytoplasm by Exportin-5 and finally processed by the RNase III Dicer into mature miRNAs. In the cytoplasm, miRNAs associate with RNA-

induced silencing complex (RISC) and target mRNA transcripts to degradation or suppress their translation<sup>199-201</sup>.

There are several types of non-coding RNA. Some are involved in epigenetic regulation such as X inactivation, dosage compensation, imprinting, and Polycomb silencing. Because microRNAs are closely associated with small interfering RNA, and the latter has been associated with DNA methylation and histone modifications, microRNAs have been considered to be “epigenetic” factors<sup>200, 202, 203</sup>. In addition, microRNAs are involved in DNA methylation in *Arabidopsis thaliana*<sup>204</sup> and might be involved in regulating the histone deacetylase HDAC4 in murine developing cartilage<sup>205</sup>. At this stage, however, the epigenetic role of microRNA is predominantly indirect.

There is a rapidly growing body of evidence that microRNAs play a role in gene regulation during development, cellular differentiation, aging and immunity<sup>201, 206-212</sup>. Also, microRNAs are implicated in oncogenesis; and in the age-related diseases Parkinson’s disease and Alzheimer’s disease; and rheumatoid arthritis<sup>201, 212-214</sup>. More recently, miR-155 and miR-146 were identified in synovium from RA and OA patients<sup>215-217</sup>. miR-146 is upregulated by TNF- $\alpha$  and IL-1 $\beta$  but its function remains unknown. miR-155 is upregulated by cytokines and Toll-like receptor ligands, and it might work modulating MMP response by RA synovial fibroblasts<sup>215, 216</sup>.

### **The epigenomic landscape**

The recent development of high throughput technologies (ChIP-chip and ChIP sequencing)<sup>170, 218</sup> have allowed researchers to identify and map epigenetic marks across the entire genome, to compare epigenetic features of specific regions between different

tissues, and to associate epigenetic alterations with gene transcriptional states. Analyses of several human tissues, placenta, sperm, mouse embryonic stem cells, neural progenitor cells, embryonic fibroblasts and several cell lines in addition to cells in different stages of differentiation, led to identification of certain epigenetic features:

- 1- in general, promoter methylation correlates negatively with gene expression at high-, medium- and low-CpG density promoters;
- 2- promoter hypomethylation and gene body methylation positively correlates with gene expression;
- 3- promoters with few CpGs can be active and methylated;
- 4- exons are more highly methylated than introns, and sharp transitions occur at exon–intron boundaries, suggesting that differential methylation is involved in splicing;
- 5- about 25% of methylation found in embryonic stem cells is not in a CpG context and is lost during cellular differentiation;
- 6- while overall methylation decreases, developmentally regulated and pluripotency-associated genes become methylated during stem cell differentiation;
- 7- methylation of CpG island shores (regions of lower CpG density near (~2 kb) CpG islands) of promoter-associated CpG islands correlates with transcriptional inactivation;
- 8- most tissue-specific DNA methylation is found in CpG island shores, not at CpG islands;
- 9- unmethylated promoters are associated with H3K4 trimethylation and are actively transcribed;



- 10- H3K4 trimethylation, H3K4 dimethylation, and H3 acetylation are localized to the transcriptional start sites of genes;
- 11- H3K27me3 is associated with inactive promoters;
- 12- “bivalent domains” (H3K4me3 active mark found together with the H3K27me3 repressive mark) are present at promoters of developmentally important genes in embryonic stem cells and are lost during cell differentiation;
- 13- H3K9 and H3K27 trimethylation are usually associated with heterochromatin formation and silencing of transcription through the Polycomb group proteins;
- 14- H3K9 trimethylation and H3K20 trimethylation are found at satellite, telomeric and active long-terminal repeats;
- 15- active enhancers are marked by H3K4 monomethylation and H3K27acetylation;
- 16- H3K36 trimethylation marks exons;
- 17- cell line epigenetic profiles do not correspond to the epigenetic profiles of their primary tissue; <sup>169, 219-229</sup>

It is becoming increasingly evident that multiple epigenetic alterations function in concert with RNA polymerase and associated core transcription factors to regulate transcriptional activity across the genome.

### **DNA methylation, cellular differentiation and phenotypic stability**

Several studies have demonstrated the importance of epigenetics in stem cell differentiation and lineage commitment. For example, several germline-specific genes are methylated in fibroblasts but not in sperm <sup>230</sup>, suggesting that DNA methylation is involved in repression of germ-line specific genes in somatic cells, and participates in the

epigenetic regulation of gene expression during cell differentiation<sup>231-233</sup>. Moreover, genome-wide screens have provided information on differential methylation in different types of tissues<sup>232, 234-237</sup>. The largest study in this field was published in 2008 by Rakyan and colleagues<sup>224</sup>. They used Methylated DNA Immunoprecipitation (MeDIP) associated with microarrays to investigate the methylation and gene expression profile of 13 normal somatic tissues, placenta, sperm and a lymphoblastic cell line. They identified several differentially methylated regions (tDMRs) which regulate tissue-specific gene expression.

### **DNA methyltransferase inhibitors**

The DNA methyltransferase inhibitors are derivatives of 5-azacytidine (5-aza). 5-aza is a cytidine analog modified at position 5 that cannot be methylated. It was originally synthesized by Piskala and Sorm in 1964 with the objective to treat acute myelogenous leukemia<sup>238</sup>. Nowadays, 5-aza and several other cytidine analogues, such as 5-aza-2-deoxycytidine (5-aza-CdR), zebularine, and decitabine, among others, are used to treat several neoplastic diseases<sup>183, 239</sup>.

5-aza has its effect on genomic methylation by functioning as a cytidine analog during DNA replication. Generally, only 5% of cytidines are substituted by 5-aza, but this degree of substitution is sufficient to induce a global decrease in genomic DNA methylation (80-85%), since incorporated 5-aza covalently binds Dnmt1<sup>240-243</sup>. This effectively sequesters the enzyme and leads to a global reduction in genomic methylation<sup>242, 243</sup>.

There are other mechanisms by which 5-aza might influence chondrocyte activity. DNA/Dnmt adducts triggers a DNA damage response which leads to p53 activation, increased p21Waf1/Cip1 expression, and cell cycle inhibition<sup>244</sup>. Zhu *et al*, 2004<sup>245</sup> used deletion analyses and site-directed mutagenesis of the p21 promoter to demonstrate that 5-aza-CdR induction of p21Waf1/Cip1 expression requires two p53 binding sites in the p21 promoter. Comet assays and bisulfite sequencing were used to demonstrate that 5-aza-CdR-induced p21Waf1/ Cip1 expression was dependent on DNA damage but not on DNA demethylation.

Others have also demonstrated that 5-aza-CdR has replication-independent effects<sup>246-249</sup>. Firstly, 5-aza-CdR is able to induce Dnmt1 degradation by the proteosomal pathway<sup>249</sup>. Secondly, Dnmt1 can control DNA methylation in a replication-independent manner since it binds chromatin throughout G2 and M phases<sup>248</sup>. Finally, 5-aza can be incorporated into RNA, resulting in the inhibition of RNA and protein synthesis independently of any effect on the genome<sup>246, 247</sup>.

Therefore, 5-aza and its analogs can influence cellular systems through their ability to decrease global methylation in replication-dependent or –independent manners, through DNA damage response, or through inhibition of mRNA and protein synthesis. These alternative mechanisms are particularly pertinent to the actions of 5-aza in chondrocyte populations, since phenotypically stable chondrocyte culture models (low/no serum-suspension/non-adherent) do not support sustained cell proliferation.

DNA methyltransferase inhibitors have been used to experimentally address the effects of DNA methylation on cellular differentiation and phenotypic stability. For instance, DNA methyltransferase inhibitors induce mouse embryo fibroblasts to

differentiate into muscle, adipose tissue and chondrocytes<sup>250-252</sup>. In addition, 5-aza-CdR prevents differentiation of spermatogonia into spermatocytes in mouse<sup>253</sup>, which suggests that DNA methylation is important for terminal differentiation of spermatocytes. In contrast, the treatment of articular chondrocytes with DNA methyltransferase inhibitor alleviates molecular constraints that prevent these cells from achieving terminal maturation<sup>254</sup>.

## **EPIGENETICS AND CHONDROCYTE PATHOBIOLOGY**

### **Epigenetics and aging**

During aging there is a change in methylation pattern leading to methylation of CpG islands and global demethylation of the genome. Methylation of CpG islands is associated with suppression of tumor suppressor genes and oncogenesis<sup>200, 255</sup>. In addition, histone deacetylase activity is decreased in aged tissues and it seems to have an important role increasing lifespan, as demonstrated in yeast and mice<sup>256</sup>. Moreover, H4K20 trimethylation, a repressive marker, has also been shown to be increased in aging<sup>257</sup>. MicroRNAs have also been implicated in age-related diseases, ie Parkinson's disease and Alzheimer's disease<sup>214</sup>. Therefore, it seems that alterations in the epigenetic status during aging, leads to redistribution of heterochromatin, changes in pattern of gene expression, increases genomic instability and impairs cell function.

Several age-related diseases, such as atherosclerosis, neurologic disorders, cancer, and autoimmune diseases, have been linked to altered cell function due to epigenetic alterations<sup>258</sup>. Moreover, cells obtained from elderly people present epigenetic

alterations similar to those observed in Progeria, a syndrome characterized by an accelerated aging phenotype<sup>257</sup>. This suggests that epigenetics influence the process of aging itself.

Some studies suggest that certain changes during OA might be related to epigenetic changes in gene regulation<sup>61, 259, 260</sup>. The extent to which epigenetics play a role in OA, and the extent to which these changes are related to epigenetic changes due to senescence, remains unknown.

### **Epigenetics and rheumatoid arthritis**

Rheumatoid arthritis (RA) is an auto-immune disease characterized by chronic inflammation of several joints, synovial hyperplasia and cartilage destruction.<sup>261</sup> MMP-1, a cartilage degradative enzyme, is increased in RA synovial fibroblasts (RASFs). Maciejewska-Rodrigues and colleagues demonstrated that epigenetics play a role in the regulation of MMP-1 expression in RA. The MMP-1 promoter is hyperacetylated in RASFs compared to OA synovium fibroblasts; and transcriptional repression depends on HDAC4<sup>262</sup>. Further, global HDAC activity was decreased in synovium from RA patients when compared to synovium from OA patients<sup>263</sup>. In addition, RASFs show global hypomethylation of the genome when compared to OA synovial fibroblasts<sup>264, 265</sup>. Activation of LINE-1 (long interspersed nuclear element-1) retrotransposons by hypomethylation might be involved the pathogenesis of the activated phenotype of RASFs. Normal synovial fibroblasts treated with the Dnmt inhibitor 5-aza-CdR changed their cellular profile into an RASF-like phenotype<sup>264</sup>.

MicroRNAs also appear to be involved in RA pathogenesis. miR-155 and miR-146 are present in higher amounts in RA synovium than in OA or normal synovium and are upregulated by TNF- $\alpha$  and IL-1 $\beta$  treatments<sup>215, 216</sup>. RA synovial fibroblasts treated with TNF- $\alpha$ , IL-1 $\beta$ , lipopolysaccharide or bacterial lipoprotein upregulated miR-155 and miR-146. miR-155 decreased MMP-3 expression and reduced the induction of MMP-3 and MMP-1 by TNF- $\alpha$ , IL-1 $\beta$ , lipopolysaccharide or bacterial lipoprotein<sup>216</sup>. This suggests that microRNAs might be involved in the modulation of inflammatory response by RASFs in rheumatoid arthritis.

### **Epigenetics and chondrocyte biology**

Several recent studies have demonstrated the importance of epigenetic gene regulation in chondrocyte development and homeostasis.

Sox-9 is a transcription factor master regulator of several chondro-specific genes<sup>3-11</sup>. Tsuda and co-workers demonstrated that histone acetyltransferase, CBP/p300, associates with Sox-9 to regulate transcription of the Col II gene in chondrocytes<sup>266</sup>. CBP, p300 and Sox-9 were overexpressed in the chondrosarcoma cell line SW1353, resulting in increased Col2a1 promoter activity. Co-immunoprecipitation and Western Blot analyses showed that Sox-9 interacts with HA-CBP or HA-p300. Moreover, chromatin immunoprecipitation demonstrated that p300 binds to the collagen type II promoter region. Disruption of the CBP/Sox9 interaction inhibited MSC chondrogenesis<sup>266</sup>. Taken together, these findings demonstrate that the CBP/p300 are co-activators of Sox9 in the regulation of chondro-specific genes and during *in vitro* chondrogenesis.

Furumatsu and colleagues, also from the Asahara lab, investigated the mechanisms by which p300 acts as Sox-9 co-activator. Chromatinized DNA templates were used to assess Sox-9 dependent expression *in vitro*. p300 increased Sox-9 dependent transcription on chromatin and correlated with hyperacetylated histones. Moreover, trichostatin A, a histone deacetylase inhibitor, stimulated expression of Col II and aggrecan, and increased nucleosomal acetylation around the Sox-9 binding site in chondrocytes<sup>267</sup>. These results support a model whereby the gene-specific factor Sox-9 interacts with chromatin-remodeling factors to activate Col II transcription. This model likely represents a more general trans-regulatory mechanism by which tissue-specific transcription factors recruit non-specific chromatin-modifying agents to lineage-associated genes.

HDAC4 is a class II histone deacetylase and is specifically involved in regulation of chondrocyte hypertrophy. HDAC4 null mice exhibit a premature chondrocyte hypertrophy and ossification phenotype, characteristics similar to mice that overexpress RUNX-2 in chondrocytes. Corroborating this association, constitutive expression of HDAC4 mimics the phenotype seen in RUNX-2 knockout mice<sup>268</sup>. *In vitro* studies showed that HDAC4 interacts with Runx2, inhibits Runx2 DNA binding and decreases histone acetylation of RUNX-2 promoter<sup>268</sup>. HDAC4 also interacts with and represses the transcription factor MEF2 (myocyte enhancer factor 2) that is also required for chondrocyte hypertrophy. PTHrP mediates the dephosphorylation and consequent nuclear localization of HDAC4 in pre-hypertrophic chondrocytes, facilitating MEF2 repression by HDAC4<sup>269</sup>. Over-expressed HDAC4 had similar hypertrophy-inhibitory effects in a chondrogenesis model utilizing synovial derived MSCs<sup>270</sup>. These findings indicate that

HDAC4 functions as a negative regulator of chondrocyte hypertrophic differentiation by suppressing the activities of Runx2 and MEF2, a transcription factors required for hypertrophy. The extent to which HDAC4 affects this regulation through histone modifications, as opposed to altering the acetylation status of non-histone substrates, has not yet been clarified.

Experimental evidence exists that indicates HDAC activity is also necessary to support collagen type II expression in articular chondrocytes, and permit re-differentiation of (monolayer) de-differentiated chondrocytes<sup>271</sup>. In this study, HDAC inhibitors trichostatin A (TSA) and PXD101 inhibited collagen type II expression by articular chondrocytes and prevented re-differentiation of dedifferentiated chondrocytes. Knockdown studies supported the conclusion that HDACs inhibit collagen type II expression by upregulating Wnt-5A, a signaling protein known to suppress the chondrocytic phenotype. In addition, chromatin immunoprecipitation showed an increase in histone acetylation of the Wnt-5A promoter in the presence of HDAC inhibitors<sup>271</sup>.

DNA methylation also plays a role in regulating the chondrocytic phenotype. Phenotypically stable, chick articular chondrocytes, cultured as monolayers and treated with the DNA methyltransferase inhibitor, 5-aza, undergo hypertrophy, expressing Col X, Ihh, and ALP<sup>254</sup>. Bmp-2 treatment increased Col X and ALP expression by chondrocytes treated with 5-aza. On the other hand, Tgf- $\beta$  and PTHrP treatment reduced Col X expression. To investigate the mechanisms involved in chondrocyte hypertrophy following 5-aza treatment, expression of the BMP Smads (1 and 5), the TGF- $\beta$  Smads (2 and 3), and the Smad2/3-degrading ubiquitin E3 ligase Smurf2 were assessed by Western Blot analyses. 5-aza treated chondrocytes had increased levels of Smad1, 5, and Smurf2



and decreased levels of Smad2 and 3 proteins. Smurf2-dependent degradation of Smad2 and 3 was demonstrated by pulse-chase experiments to measure ubiquitination and half life <sup>254</sup>. Altogether, the data suggest that differential DNA methylation is involved in the regulation of TGF- $\beta$  and BMP signaling interactions to control lineage commitment of chondrocyte populations. Accepting this, no data were presented in this study that demonstrated a direct link between altered methylation states and consequent phenotypic alterations.

In a related study, fetal bovine chondrocytes cultured as monolayers responded to 5-aza treatment in a similar manner, expressing hypertrophic markers. Cells exposed to 5-aza for 48 hours decreased collagen type II expression and up-regulated hypertrophic markers such as ALP and Col X <sup>272</sup>. These cells also increased expression of PTHrP and the PTHrP receptor. These genes are markers of pre-hypertrophic chondrocytes, and are not expressed by hypertrophic cells. It is likely that the observed changes in gene expression in this study reflect a range of phenotypic states in the sample population, rather than a consistent and uniform response to 5-aza. In addition, human articular chondrocytes cultured in alginate beads undergo phenotypic changes that resemble hypertrophy, following 5-aza treatment, with modest increases in Col X and ALP expression and down-regulation of collagen type II expression. Chondrocyte size and apoptosis were also increased following 5-aza exposure. The authors suggested that this model could be applied to study the cellular mechanisms of OA <sup>273</sup>.

It is worth mentioning that there were no attempts to ensure that 5-aza worked as a demethylating agent in the experiments above described. 5-aza also induces cellular responses through the DNA damage response, which leads to p53 activation,

p21Waf1/Cip1 up-regulation and cell cycle arrest<sup>245</sup>. This mechanism can explain the increased chondrocyte apoptosis observed in Ho's study, for instance. It is a distinct possibility that other non-epigenetic mechanisms might be involved in the 5-aza mediated phenotypic shifts described above.

### **Epigenetics and osteoarthritis**

HDAC inhibitors have been investigated as potential therapeutic agents for the treatment of osteoarthritis<sup>274</sup>. Young *et al.* (2005) utilized a bovine nasal cartilage explant model to induce cartilage matrix degradation by a combination of IL-1 $\alpha$  and oncostatin M. Explants co-treated with HDAC inhibitors (trichostatin A or sodium butyrate) exhibited decreased cartilage matrix degradation by blocking the induction of MMP 1 and 13 and also inhibiting ADAMTs 4, 5 and 9 mRNA expression<sup>275</sup>. The authors suggest that HDAC inhibitors could be used as chemo/therapeutic agents in OA due to their chondroprotective effects. Accepting these conclusions, the study did not directly address histone associated mechanisms involved in MMPs or ADAMTs suppression.

Several recent studies by Roach and colleagues have established a link between altered CpG methylation and expression of degradative enzymes by articular chondrocytes in OA cartilage<sup>260, 276-278</sup>. Roach and co-workers used immunohistochemistry to assess MMP-3, -9, and 13 and ADAMTS-4 expression by human OA chondrocytes. All four enzymes were present in late OA specimens and co-localized to chondrocyte clusters. The methylation status of these CpG sparse promoters were assessed by methylation-sensitive restriction enzyme digestion followed by PCR

amplification. For all four enzymes, the overall demethylation percentage was increased and some sites were preferably demethylated in OA samples<sup>276</sup>. Similar results were obtained in subsequent experiments<sup>277, 278</sup>. The authors suggest that demethylation of certain CpG dinucleotides is responsible for epigenetic de-silencing and expression of these enzymes in OA, although no experiments were carried out to test whether these specific sites were important for gene expression. Also there was no reference to whether the CpG sites in question lie in areas of transcription factor recognition. Therefore, more studies need to be done to prove that the observations above are responsible for epigenetic gene regulation of the degradative enzymes in OA or to find out that these are mere stochastic events resultant of activation of these enzymes by other means.

IL-1 $\beta$  is a key cytokine involved in inflammatory responses and upregulation of degradative enzymes in osteoarthritis. Roach's laboratory demonstrated that 5-aza-CdR upregulates IL-1 $\beta$  by human articular chondrocytes<sup>260</sup>. Bisulfite conversion and sequencing showed that IL-1 $\beta$  was preferably demethylated at CpG site 299 bases upstream of the start site. Interestingly, treatment with cytokines (TNF- $\alpha$  plus oncostatin M) was more effective than 5-aza-CdR in increasing IL-1 $\beta$  levels and also correlated with more -299 CpG demethylation in the IL-1 $\beta$  promoter region. Since TNF- $\alpha$  and oncostatin M do not have known demethylation properties, it is probable that other mechanisms are involved in active demethylation of the IL-1 $\beta$  promoter. The authors suggest that NF- $\kappa$ B might play a role in demethylation of IL-1 $\beta$  promoters<sup>279</sup>, since the -299 CpG site is within/adjacent a canonical NF- $\kappa$ B binding site.

Work done by Simopoulou and others demonstrated that leptin might act as a link between obesity and osteoarthritis. Levels of leptin are increased in synovial fluid of

osteoarthritic joints. In addition, treatment of chondrocytes with leptin leads to upregulation of IL-1 $\beta$ , MMP-9 and 13 protein levels<sup>280</sup>. In addition, the same group found that epigenetic mechanisms are involved in the regulation of leptin expression in osteoarthritic chondrocytes. DNA bisulfite sequencing was used to demonstrate that the leptin promoter is methylated in normal chondrocytes but demethylated in severe OA. Moreover, the use of small interference RNA against leptin down regulated MMP-13 expression. Administration of 5-aza-CdR to normal chondrocytes demethylated the leptin promoter, upregulating leptin and its downstream target gene, MMP-13. Treatment with the HDAC inhibitor, TSA, also increased leptin expression by normal chondrocytes and correlated with H3K9 and K14 acetylation at the leptin promoter<sup>259</sup>.

Not all changes in gene expression observed during osteoarthritis are associated with changes in promoter methylation profile. As examples, p21<sup>281</sup> and aggrecan<sup>282</sup> down regulation in OA chondrocytes do not appear to be regulated by hypermethylation of the promoter sequences. Consequently, gene activation or repression is the result of interactions between transcription factors and epigenetic control.

The majority of articles mentioned above do not show direct evidence that 5-aza exerts its effects on chondrocyte behavior by decreasing methylation at regulatory regions of target genes. Given the global epigenomic effects of 5-aza administration, mechanistically proving a functional consequence to specific gene expression is experimentally challenging, to say the least.

Considering the increasing amount of information on epigenomic contributions to aging related disease, and the current amount of data associating epigenetic pathways in chondrocyte differentiation, phenotypic maintenance and the progression of OA, the

following studies were carried out to address the actions and mechanisms of 5-azacytidine and genomic methylation in regulating of collagen type II expression by osteoarthritic chondrocytes (Chapter 3), in the phenotypic alterations that develop in OA chondrocytes (Chapter 4) and in the phenotypic differences between articular and endochondral chondrocyte populations (Chapter 5).

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# **CHAPTER 3: PHENOTYPIC EFFECTS OF THE DNA METHYL-TRANSFERASE INHIBITOR, 5-AZACYTIDINE, ON ARTICULAR CHONDROCYTES**

## **INTRODUCTION**

Articular cartilage is a specialized tissue that covers the articulating surfaces of bones. It is avascular and rich in extracellular matrix composed predominantly of water, collagen type II (Col II) and proteoglycans. Collagen fibrils anchored in the calcified cartilage plate provide the cartilage with tensile strength and resistance to surface shear, while the aggrecan: proteoglycan complexes bind water molecules and generate resistance to compressive loads <sup>1, 2</sup>. Under normal circumstances, articular chondrocytes do not undergo hypertrophic maturation. Instead, articular chondrocytes persist throughout life in a nominally pre-hypertrophic state, characterized by the expression of Col II and aggrecan (Agg), with little or no expression of hypertrophic-specific genes such as collagen type X (Col X) and alkaline phosphatase (ALP) <sup>3</sup>.

The mechanism by which articular chondrocytes avoid hypertrophy is still not completely understood. There are several studies indicating that transforming growth factor- $\beta$  (TGF- $\beta$ ) <sup>4-6</sup>, bone morphogenetic proteins (BMP) <sup>7-9</sup>, parathyroid hormone related peptide (PTHrP) <sup>10-13</sup> signaling pathways, in conjunction with transcriptional factors <sup>14-22</sup> are crucial to maintenance of articular cartilage and articular chondrocytic phenotype. Interference with these signaling pathways leads to pathological changes that resemble osteoarthritis (OA) <sup>4, 5, 7, 13</sup>.

OA is a non-inflammatory disease characterized by progressive articular cartilage degradation<sup>23</sup>, calcification<sup>24</sup>, and subchondral bone alterations<sup>25</sup>. The disease is notable for the relative absence of neutrophil and macrophage infiltration, but high levels of inflammatory cytokines are produced by synoviocytes and chondrocytes<sup>23, 26, 27</sup>, resulting in “inflammation-like” cell responses. Although OA is predominantly characterized by loss of extracellular matrix homeostasis, the disease also causes distinct phenotypic alterations in the resident chondrocyte population. Articular chondrocytes undergo phenotypic changes resembling the transitions that occur during hypertrophic differentiation of endochondral lineage chondrocytes<sup>28</sup>. Articular chondrocytes down-regulate Col II and Agg expression<sup>29, 30</sup> and begin to express Col X<sup>3, 31-34</sup>. This has been demonstrated by microarray comparisons<sup>35</sup> and differential Col X immunostaining in OA and normal cartilage<sup>33, 36</sup>. Aberrant expression of other hypertrophic markers such as ALP and osteocalcin (OCN) has also been linked to OA<sup>36, 37</sup>.

In recent years, the role of epigenetic regulation in the control of cellular phenotype has been increasingly recognized. Epigenetics refers to “mechanisms that initiate and maintain patterns of gene expression and gene function in a heritable manner without changing the sequence of the genome”<sup>38</sup>. Epigenetics impact gene regulation through DNA methylation, histone protein modifications and consequent chromatin organization. Overall, DNA methylation of CpG islands is associated with histone hypoacetylation that collectively repress transcription. DNA methyltransferases methylate cytidines at CpG dinucleotide sites. This modification can interfere with the recognition and attachment of DNA binding proteins to genomic cis elements. Additionally, methyl-CpG binding proteins are recruited to methylated CpGs (mCpG)



and, in turn, recruit histone deacetylases (HDACs), histone methyltransferases (HMTs) and chromatin-remodeling factors to methylated regions. These modifications condense chromatin structure, sequestering potential binding sites from transcriptional activators and restricting occupancy. As a consequence, transcription is repressed<sup>39</sup>. However, promoter occupancy does not depend solely in its epigenetic state. Transcription activation and repression are also regulated by competitive binding of promoter, enhancer and silencer elements<sup>40-45</sup>. Therefore, activation or repression of a gene is a collective consequence of the interplay of transcription factors and epigenetic constraints.

Epigenetic processes are involved in regulation of germline-specific genes, tissue-specific genes, imprinting, and aging<sup>38</sup>. A growing body of evidence indicates that epigenetics is also critical in a range of disease processes. Cancer cells are often globally hypomethylated, although specific tumor suppressor genes are hypermethylated and transcriptionally suppressed<sup>46</sup>. Several studies have demonstrated the importance of epigenetics in stem cell differentiation and lineage commitment<sup>47-50</sup>. In addition, genome-wide screens have documented distinct methylation profiles in specific tissues<sup>48, 51-54</sup>, implicating CpG methylation in lineage commitment and phenotypic maintenance.

Several recent studies have demonstrated the importance of epigenetic gene regulation in chondrocyte development and homeostasis. Vega *et al* (2004)<sup>55</sup> demonstrated that HDAC4 functions as a negative regulator of chondrocyte hypertrophic differentiation by suppressing the activities of Runx2, a transcription factor required for hypertrophy. HDAC activity is also necessary to support collagen type II expression in articular chondrocytes, and permit re-differentiation of (monolayer) de-differentiated

chondrocytes<sup>56</sup>. In this context, HDAC activity was linked to suppression of Wnt-5A expression, a protein known to suppress the chondrocytic phenotype.

DNA methylation has been indirectly implicated in chondrocyte differentiation. The DNA methyltransferase inhibitor, 5-azacytidine (5-aza), induced expression of hypertrophic markers in chick articular chondrocytes<sup>57</sup>, suggesting that epigenetic pathways regulate the lineage commitment of chondrocyte populations. Cheung *et al* (2001)<sup>58</sup> observed similar results in bovine fetal epiphyseal chondrocytes treated with 5-aza.

A series of studies from the Asahara lab have established that the histone acetyltransferase, CBP/p300, associates with Sox-9 to regulate transcription of the Col II gene in chondrocytes<sup>59</sup>. Moreover, trichostatin A, a histone deacetylase inhibitor, stimulates expression of Col II and Agg, and increases nucleosomal acetylation around the Sox-9 binding site<sup>60</sup>. Taken together, these results support a model whereby the gene-specific factor Sox-9 interacts with chromatin-remodeling factors to activate Col II transcription.

Epigenetic pathways have also been implicated in the pathogenesis of OA. HDAC inhibitors (trichostatin A or sodium butyrate) decreased cartilage matrix degradation by blocking the induction of matrix metalloproteases (MMP) 1 and 13 and also inhibiting ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs) 4, 5 and 9 mRNA expression by bovine nasal cartilage explants co-treated with cytokines<sup>61</sup>. In addition, Roach and others, have linked osteoarthritic changes in articular chondrocyte to demethylation of specific CpG dinucleotides in MMP-3,9,13 and ADAMTS-4<sup>62-64</sup> promoters that result in epigenetic de-silencing of these genes in OA. In addition,

epigenetic mechanisms are also involved in regulating leptin expression in osteoarthritic chondrocytes <sup>65</sup>. The leptin promoter is methylated in normal chondrocytes but demethylated in severe OA cells. Leptin expression activates MMP-13 in chondrocytes <sup>65</sup>. Furthermore, treatment of normal chondrocytes with 5-aza-2-deoxycytidine (5-aza-CdR) demethylates the leptin promoter, and upregulates expression of leptin and its downstream target MMP-13 <sup>65</sup>. Ho, *et al* (2006) <sup>66</sup> showed that human articular chondrocytes cultured in alginate beads and treated with 5-aza undergo phenotypic changes that resemble hypertrophy; somewhat analogous to the phenotypic changes that occur in OA.

A number of age-related diseases, including atherosclerosis, neurologic disorders, cancer, and autoimmune diseases, have been linked to altered cell function due to epigenetic alterations <sup>67</sup>. The clinical incidence of osteoarthritis is increased in older individuals and, as discussed above, upregulation of several catabolic enzyme genes during OA appears to be linked to demethylation of their promoters. In addition to the up-regulation of inflammatory cytokines and degradative enzymes in OA, the expression of critical ECM genes, particularly the major cartilage-specific collagen type II, is reduced in OA chondrocytes. In this study we assessed the effect of 5-aza administration to OA chondrocytes on collagen type II expression, determined the CpG methylation status of the collagen type II promoter and Sox 9 enhancer sequences in chondrocytes and non-chondrocytic cell types and investigated whether expression of collagen type II in OA chondrocytes is linked to the methylation status of its promoter/enhancer regions.

## **METHODS**

### **Sample collection**

Human articular cartilage and bone were obtained from donors following total knee replacement procedures at Carle Clinic, Urbana, IL. This project was approved by both the University of Illinois Ethics Committee and the Carle Clinics Ethics Committee. Informed consent was obtained from the patients before surgery.

The articular cartilage specimens presented with variable degrees of degeneration, both between and within specimens. Therefore, articular cartilage was collected according to its macroscopic characteristics, i.e. samples that were not fibrillated were considered “normal”; whereas cartilage samples with surface irregularities were considered to be overtly osteoarthritic. Severely degenerative tissues and osteophytic cartilage were excluded from the collections. Cancellous bone was harvested from the central region of the proximal tibia fragment. One to two grams of cartilage and bone were snap frozen in liquid nitrogen and stored at -80°C for until needed for DNA isolation.

### **Chondrocyte isolation**

Articular cartilage specimens were diced, washed in phosphate buffered saline (PBS) (HyClone, Logan, UT) and transferred to 500-mL Ehrlenmeyer flasks. The cartilage explants were pre-digested in 0.3% pronase (Roche Diagnostics Corporation, Indianapolis, IN) reconstituted in Opti-MEM (Gibco, BRL-Life Technologies, Grand Island, NY) supplemented with penicillin G sodium (200 units/mL) streptomycin (200

µg/mL) (Gibco), 2.5 µg/mL of amphotericin B (Cellgro, Mediatech, Inc, Herndon, VA) and 2% of fetal bovine serum (Gemini Bio products, West Sacramento, CA), at 10 mL/g of cartilage. The flasks were placed in a C24 shaking incubator (New Brunswick Scientific, Edison, NJ) at 180 rpm at 37°C for an hour. After one hour, the pre-digestion solution was aspirated, the explants were rinsed with PBS and were resuspended in 0.15% collagenase A (10 mL per gram of tissue; Roche) reconstituted in Opti-MEM supplemented with penicillin G sodium (200 units/mL) streptomycin (200 µg/mL), 2.5µg/mL of amphotericin B and 2% of fetal bovine serum was added to the flask for the overnight digestion in the C24 shaking incubator at 180 rpm at 37°C.

After the overnight digestion, the cell suspension was filtered through a 40-micron filter (Nalgene Company, Fisher Scientific, Pittsburgh, PA) into 50-mL Falcon tubes (Fisher Scientific). The flask and filter were rinsed with PBS to recover any residual cells. After filtration, the cells were pelleted by centrifugation (Centrifuge 5810 R, Eppendorf, Westbury, NY) for 10 minutes at 290 rcf. The supernatant was aspirated and the cell pellet was re-suspended in PBS and pelleted following the same protocol to assure removal of residual collagenase. Finally, the cells were resuspended in 10-20 mL of Opti-MEM for cell counting and viability assessment.

### **Cell count and viability**

The cell numbers were calculated by counting chondrocytes in an aliquot of the resulting suspension using a hemacytometer (Fisher Scientific) and inverted light microscope (Fryer Co. Inc., Huntley, IL). Two vials containing a 1:10 dilution of the cell suspension and 10% Trypan blue dye (Gibco) were prepared. The samples were mixed

and 10  $\mu$ L were added to each side of the hemacytometer. Dead and live cells were counted in a total of 9 (1 mm<sup>2</sup>) squares in each side. The total number of cells in the cell suspension was obtained by the following formula:

$$\# \text{ of cells} = \# \text{ of cells counted} / 18 (\# \text{ of } 1 \text{ mm}^2 \text{ squares counted}) \times 10 (\text{cell dilution}) \times 10 \text{ or } 20 (\text{total volume of the cell suspension}) \times 10^4$$

### **Chondrocyte culture conditions**

Articular chondrocytes (3 to 4 million cells per well) were cultured as non-adherent aggregates using a defined serum-free culture medium (Opti-MEM, Gibco) in hydrogel-coated, ultra low attachment culture plates (six-well plates (10 cm<sup>2</sup> wells)) (Corning Costar, Pittsburgh, PA) or as monolayers (50,000 cells per cm<sup>2</sup> in 100 mm<sup>2</sup> culture plates (Corning Costar), in 10mL of medium). Opti-MEM is a proprietary medium, based on alpha MEM and supplemented with insulin, transferrin and selenous acid (ITS). It was originally developed for low-serum growth of hybridoma cultures, but also supports chondrocyte cultures very well<sup>68, 69</sup>. The media was supplemented with ascorbic acid (Sigma-Aldrich, St. Louis, MO) (50  $\mu$ g/mL) penicillin G sodium (100 units/mL) and streptomycin (100  $\mu$ g/mL). The culture medium was changed every two to four days depending on cellular metabolism. Cultures were maintained at 37°C, in 95% air/ 5% CO<sub>2</sub> in a humidified incubator (Fisher Scientific). After 24 hours of seeding, the chondrocytes were treated or not with 10  $\mu$ M of 5-azacytidine (Sigma-Aldrich) for 4 days (non-adherent aggregates) or 15 days (monolayers). If the media needed to be changed before collection, a freshly prepared 5-aza solution was added to the media. This

treatment interval provides ample opportunity for at least one round of cell division, and consequent 5-aza incorporation.

The chondrocyte aggregates were collected by transferring the aggregates and media from each well into a 15-mL Falcon tube. The monolayer cultures were washed with cold PBS then immediately lysed in TRIzol (see below). The lysates were aspirated from the plates and transferred to 15-mL Falcon tubes. For DNA collection, monolayers were washed with PBS, scraped directly off the plates and transferred to 15-mL Falcon tubes. The 15-mL tubes were centrifuged in a refrigerated centrifuge (Eppendorf) at 290 rcf for 10 minutes. After pelleting, the media were aspirated from the samples and the pelleted cells were snap frozen in liquid nitrogen and stored at -80°C until processed.

#### **Cell line culture conditions**

TC-28a4, HTB94, U2OS, SAOS-2 and HeLa cell lines were seeded at 20,000 cells/cm<sup>2</sup> in 100 mm<sup>2</sup> culture dishes (Corning Costar) and cultured as monolayers in 10 mL of DMEM (Dulbecco's Modification of Eagle Medium, Mediatech Inc., Manassas, VA) supplemented with 10% fetal bovine serum (Gemini Bio products, West Sacramento, CA) penicillin G sodium (100 units/mL) and streptomycin (100 µg/mL). The culture medium was changed every two to four days depending on cellular metabolism. Cultures were maintained at 37°C, in 95% air/ 5% CO<sub>2</sub> in a humidified incubator (Fisher Scientific). After 24 hours of seeding, the HeLa cells were treated or not with 10 µM of 5-aza (Sigma-Aldrich) for 4 days. If the media needed to be changed before collection, a freshly prepared 5-aza solution was added to the samples treated with

5-aza. This treatment interval provides ample opportunity for at least one round of cell division, and consequent 5-aza incorporation.

Samples for RNA and DNA isolation were handled as for chondrocyte monolayer cultures, above.

### **RNA isolation**

Total RNA was isolated from the chondrocyte aggregates using phenol-based dissociation agent, TRIzol<sup>®</sup> (Invitrogen Corporation, Carlsbad, CA). One milliliter of TRIzol<sup>®</sup> was added to the frozen aggregate pellet and the sample was immediately homogenized using an Ultra-Turrax T25 homogeneizer (Janke & Kunkel, IKA-Labortechnik, Staufen, Germany) for 30 seconds. The samples were then centrifuged in a refrigerated centrifuge 5810 R (Eppendorf) at 453 rcf for 10 minutes to precipitate the insoluble debris. The TRIzol<sup>®</sup> supernatants were transferred to 1.5-mL microcentrifuge tubes (Eppendorf) and 200  $\mu$ L of chloroform per milliliter of TRIzol<sup>®</sup> was added to separate the aqueous and organic phases of the lysate. After vortexing, the tubes were centrifuged in a refrigerated centrifuge (Centrifuge 5415 R, Eppendorf) at 16,100 rcf for 20 minutes at 4°C. The upper aqueous phase (approximately 400  $\mu$ L) was removed and placed in a new 1.5-mL microcentrifuge tube. During this step, great care was taken to leave the insoluble material at the interface intact. This interface material is comprised of insoluble proteins, lipids, DNA and, with chondrocyte cultures, large amounts of proteoglycans. To maximize RNA yields, the remaining aqueous volume and a small amount of the interface (400  $\mu$ L) was then transferred to a 0.5-mL microcentrifuge tube



(Fisher). These smaller tubes were then centrifuged at 16,100 rcf and an additional 100-200  $\mu$ L of aqueous phase was transferred to the 1.5-mL microcentrifuge tubes.

The RNA was precipitated from the aqueous solution by addition of 250  $\mu$ L of isopropanol and 250  $\mu$ L of 1.2 M sodium chloride/0.8M sodium citrate. This high-salt precipitation protocol minimizes co-precipitation of proteoglycans. The microcentrifuge tubes were stored overnight at -20°C to facilitate RNA precipitation. The samples were then centrifuged at 16,100 for 30 minutes at 4°C to pellet the RNA. The pellet was washed with 500  $\mu$ L of 70% ethanol to reduce the salt content of the pellet, and allowed to air dry for 10 minutes. The pellet was dissolved in diethyl pyrocarbonate (DEPC)-treated water. A 1:40 aliquot of each sample was used to quantify the nucleic acid concentration spectrophotometrically (SmartSpec<sup>TM</sup> 3000, Bio-Rad, Hercules, CA), using optic density (OD) values at 260 and 320 nM. The concentration of total RNA was calculated as follow:

$$\text{RNA in } \mu\text{g}/\mu\text{L} = \frac{\text{OD at 260 nM} - \text{OD at 320 nM} \times 40 (\text{dilution factor}) \times 40 (\text{coefficient})}{1000}$$

### **Northern blot analyses**

Four micrograms of total RNA were brought up to 90  $\mu$ L using DEPC-treated water then 10  $\mu$ L of 3 M Na acetate pH 5.2 and 300  $\mu$ L of 100% ethanol were added. The samples were stored overnight at -20°C to allow the RNA to precipitate. The samples were then centrifuged at 16,100 for 30 minutes at 4°C, the pellet was washed with 500  $\mu$ L of 70% ethanol, air-dried for 10 minutes and reconstituted in 20  $\mu$ L of sample buffer. The sample buffer constituted 600  $\mu$ L of formamide, 240  $\mu$ L of DEPC-treated water, 160  $\mu$ L of formaldehyde, 100  $\mu$ L of 10x MOPS, 100  $\mu$ L of blue dye (50% formamide, 50% of

80% glycerol and bromophenol blue) and 10  $\mu$ L of ethidium bromide. The samples were placed on ice for 5 minutes and then transferred to a 65°C water bath for 15 minutes to completely solubilize the RNA. The samples were loaded in a 1% agarose/formaldehyde gel to allow transcript size separation by electrophoresis in MOPS buffer. To maintain a constant ionic gradient during the 3-hour electrophoresis process, the gel was elevated above the surface of the electrophoresis platform using glass rods, and the buffer was re-circulated between the electrodes with a peristaltic pump (Mini-pump variable flow, Fisher Scientific). After RNA electrophoresis, the integrity of RNA samples was verified by visualization of ethidium bromide-stained ribosomal bands under ultra-violet light (High Performance Ultraviolet Transilluminator, UVP, LLC, Upland, CA). The electrophoresed RNA was transferred from the gel onto nylon membranes (GE Infrastructure, Minnetonka, MN) by overnight capillary transfer using a high-salt (10X SSC) transfer solution. After transfer, the membranes were washed in 5X SSPE at 60°C to remove adherent agarose and salts, then the transferred RNAs were immobilized to the nylon substrate by UV irradiation crosslinking (Spectrolinker XL-1000 UV crosslinker, Spectronic Corporation, Westbury, NY).

For Northern blot hybridization, membranes were re-hydrated, rinsed in 5xSSPE and then pre-hybridized at 65°C in a rotating hybridization oven for 1-2 hours in pre-hybridization solution (5X SSPE, 5 X Denhardt's solution (Eppendorf), 0.25% SDS (Fisher Scientific), 10% dextran sulfate (Fisher Scientific)) and 150  $\mu$ g/mL denatured salmon sperm DNA (Invitrogen). Isotopic probes were prepared by random-primed labeling, using a commercial labeling kit (Prime-it® II, Stratagene, Cedar Creek, TX) and <sup>32</sup>P-dCTP (Amersham, Piscataway, NJ). The probes were purified using G-50 Sephadex

spin columns (Boehringer-Mannheim, Indianapolis, IN), denatured and then added to hybridization tubes containing the pre-hybridized membranes.

After overnight hybridization, the membranes were rinsed in 2X SSPE, 0.5% SDS. Sequential washes were done until the washing solution reached 0.5xSSPE with 0.125% SDS and the membrane background activity was minimal, as assessed by a handheld Geiger counter GSM-110 (Wm. B. Johnson & Associates, Inc., Fairlea, WV). The membranes were wrapped in Saran plastic and exposed overnight to a phosphor screen (Packard Instrument Comp, Inc., Meridan, CT) for quantitative analyses of signal intensity with Cyclone Storage Phosphoscreen (Packard). Finally, the membranes were exposed to autoradiograph film (Kodak Bio-Max MS Film, Carestream Health, Inc., Rochester, NY) for “hardcopy” recording of membrane activity. Radiograph cassettes were stored overnight at  $-80^{\circ}\text{C}$ . Details of the primers used to generate cDNAs for Northern blot probes are provided in Table 1.

### **Reverse transcription**

First strand cDNA was synthesized by reverse transcription using First Strand cDNA Superscript II (Invitrogen) and Oligo dT as per manufacturer’s instructions. Briefly, one microgram of total RNA was brought up to 10  $\mu\text{L}$  and then combined with 1  $\mu\text{L}$  of oligo (dT) (0.5 $\mu\text{g}/\mu\text{L}$ ) and 1  $\mu\text{L}$  of 10 mM dNTPs. The sample was incubated in a  $65^{\circ}\text{C}$  water bath for 5 minutes and then placed on ice for 1 minute to denature the RNA and allow binding of the oligonucleotides. The following reagents were added: 2  $\mu\text{L}$  10x First Strand Buffer, 2  $\mu\text{L}$  0.1M DTT, 2  $\mu\text{L}$  50 mM  $\text{MgCl}_2$  and 1  $\mu\text{L}$  RNase out. After a gentle mix and brief centrifugation, the sample was incubated at  $42^{\circ}\text{C}$  water bath for 2

minutes, 1  $\mu$ L (50 units) of SuperScript II Reverse Transcriptase was added. The sample was then incubated at 42°C for 50 minutes. The reaction was stopped by placing the sample in a 70°C water bath for 15 minutes. The RT reactions were diluted (1:10 ratio) with water and stored at -20°C until further use.

### **Quantitative polymerase chain reaction (qPCR)**

Quantitative PCR (qPCR) was used to compare gene expression profiles of different cartilage samples. The PCR reactions were carried out in a final volume of 25  $\mu$ L. The 2x SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) was used for these reactions. This reagent contains 100 nM KCl, 40 mM Tris-HCl, 0.4 mM of each dNTP, 50 units/mL of *Taq* polymerase, 6 mM MgCl<sub>2</sub>, and 20 nM SYBR Green I. The SYBR Green Supermix (12.5  $\mu$ L) was added to 1  $\mu$ L of the sense (10 pMol) and 1  $\mu$ L of the antisense primers. Autoclaved distilled water (5.5  $\mu$ L) and 5  $\mu$ L of cDNA template were also added. Initial denaturation step was carried out at 95°C for 3 minutes, then 45 cycles of denaturation at 95°C for 10s, annealing temperature of 62°C for 30s and polymerase extension at 72°C for 20s followed. The samples were denatured at 95°C for 1 min before starting the melting curve protocol that consisted of decreasing the temperature at 55°C for 1 minute followed by increments of 0.5°C every 10 s till 95°C was reached. The primers used for qPCR analyses are listed in Table 2. The primer combinations used in these experiments were designed for annealing at 60-62°C, however optimal annealing temperatures were determined by temperature gradient analyses spanning 54-64°C. The specificity of the PCR products was monitored by melting curve analyses, PCR fragment cloning and sequencing. Quantitative results were

accessed using the cycle threshold determined to represent the onset of the linear phase of amplification, as calculated by the thermal iCycler software (Bio-Rad). Sterile water was used as a 'no template' negative control for each of the PCR reactions as to monitor the possibility of contamination. The qPCR data were normalized to expression of the reference gene, elongation factor-1 alpha (EF1 $\alpha$ ).

### **DNA extraction**

Approximately 200 mg of powdered cartilage or bone was digested overnight in 2 mL of proteinase K digestion buffer (100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 0.5% SDS, 0.1 mg/mL of proteinase K) at 50°C. The DNA was extracted in 1 mL of phenol and 1 mL of chloroform: isoamyl alcohol (24:1). After vortexing, the sample was centrifuged at 8,100 rcf for 10 minutes in a bench top centrifuge (Denville 260 D, Denville Scientific Inc., Metuchen, NJ) to separate the organic and aqueous phases. The aqueous supernatant was transferred to a new microcentrifuge tube and 500  $\mu$ L of 7.5 M ammonium acetate and 2 mL of 100% ethanol were added. The sample was mixed by inversion of the tube and allowed to precipitate at room temperature for 5 minutes before being centrifuged at 8,100 rcf for 2 minutes. The supernatant was discarded and the pellet was washed with 70% ethanol, air dried and reconstituted in 270  $\mu$ L of TE buffer. The sample was re-precipitated overnight at -20°C using 30  $\mu$ L of 3 M sodium acetate and 900  $\mu$ L of 100% ethanol. The sample was pelleted at 8,100 rcf for 10 minutes, washed in 70% ethanol, air dried, resuspended in 200  $\mu$ L of TE buffer and quantified by UV spectrophotometry. The sample was stored at -

20°C until needed. The same procedure was used to isolate DNA from chondrocytes (1.5-8 million cells), however the reaction had the volume reduced by 50%.

### **Methylation-sensitive restriction enzyme digestion**

Methylation-sensitive restriction enzyme digestion was used to determine whether collagen type II expression by human osteoarthritic chondrocytes and the effects of 5-aza treatment correlate with methylation of cytidines at CpG dinucleotides. Thirteen primer pairs spanning approximately 1,200 bp upstream and 3,500 bp downstream of the Col II transcription start site were designed to comprehensively assess the promoter and first intronic Sox 9 enhancer sequences. For these analyses, the Col II methylation status of five human cell lines (chondrocytic TC-28a4, HTB-94, osteoblastic U2OS, SAOS-2 and non-skeletal HeLa cell lines), cartilage and bone samples from four individuals and three experiments comparing the methylation status of control and 5-aza-treated cells was assessed.

Five hundred nanograms of genomic DNA extracted from articular cartilage, bone or cells, were digested or not (control) with the methylation-sensitive restriction enzymes HpaII (New England Biolabs, Ipswich, MA) or HhaI (New England Biolabs) (Figure 1). The methylation-resistant restriction enzyme MspI (HpaII isosquizomer) (New England Biolabs) was used to control for complete digestion of the DNA. DNA digestion was carried out in a 40 µL reaction volume overnight at 37°C. EcoRV (Invitrogen) was also added to the digestion solutions to reduce the fragment size of the genomic templates. Twenty units of each enzyme were added to the digestion reactions to ensure complete digestion of the genomic DNA (One unit is defined as the amount of enzyme required to

digest 1 µg of λ DNA in 1 hour at 37°C in a total reaction volume of 50 µL). The restriction digestion was stopped by heating the samples at 65°C for 25 minutes. The samples were diluted with 40 µL of water. Primers targeting the Cyclin D1 promoter were used as controls for demethylation since previous experiments demonstrated that the Cyclin D1 promoter is unmethylated in chondrocytes (unpublished data). Primers targeting the imprinted gene H19 were used as positive controls for methylation. Primers and annealing temperatures are listed in Table 3. To screen for potential DNA contamination, sterile water was used as a 'no DNA template' control.

### **Polymerase chain reaction amplification**

Two microliters of the diluted samples (12.5 ng DNA) were used as templates for PCR reactions. The PCR reaction (25 µL) contained 2.5 µL of 10x PCR buffer (Invitrogen), 1 µL of 50 mM MgCl<sub>2</sub>, 0.5 µL of 10 mM dNTPs (Invitrogen), 10 pMol of the sense primer, 10 pMol of the antisense primer and 0.3 µL of *Taq* DNA polymerase (Invitrogen), in addition to the DNA template. The protocol for the PCR reaction was as follows: 95°C denaturation for 5 minutes, 30 cycles of denaturation at 95°C for 30 s, annealing at 62-66°C for 30 s, extension at 72°C for 20 s, followed by 1 cycle at 72°C for 10 min and 4°C indefinitely. The PCR products were separated by electrophoresis using a 2 % agarose TAE gel.

### **Band interpretation**

The gels were stained with ethidium bromide and photographed using a Kodak EDAS 290 system (Carestream Health) for band intensity comparison between control,

samples and those digested with HpaII, HhaI or MspI. Absence of bands in the MspI-digested sample indicated complete DNA digestion. Absence of bands in the HpaII- and HhaI-digested samples indicated complete demethylation of at least one CpG in the DNA template. The presence of faint bands in samples digested by HpaII or HhaI indicated methylation of the DNA in part of the cell population and a strong band indicated methylation of the tested CpG dinucleotides in most, if not all, cells of the population.

### **Bisulfite conversion and sequencing**

The methylation status of the promoter/enhancer region of the collagen type II gene was analyzed by bisulfite conversion and sequencing using articular cartilage collected from six individuals that underwent total knee replacement procedures. The articular cartilage was diced, snap frozen and stored at -80°C. After being pulverized under liquid nitrogen, genomic DNA was isolated by proteinase K digestion followed by phenol/chloroform extraction and ethanol precipitation as described above. One microgram of genomic DNA was bisulfite-converted using the EZ DNA Methylation™ kit (Zymo Research, Orange, CA) following the manufacturer's instructions. Bisulfite conversion transforms unmethylated cytidines to uridines that are further substituted by thymidines during subsequent PCR amplification. Two primer pairs were designed to span 700 bp of the promoter region (237 bp upstream of the transcription start site and 463 bp downstream of the transcription start site) and one primer pair was designed to span 298 bp of the collagen type II enhancer that contains the Sox-9 binding site. The following guidelines were used to design the primers:

- 1) All non-CpG cytidines were considered to be converted to thymidines



2) Between three and four non-CpG cytidines (thymidines after conversion) were included in each primer, to select for fully converted templates

3) Primers did not span CpG dinucleotides

4) Each primer pair span between 200 and 500bp. As a consequence, several primer pairs were used to comprehensively interrogate the methylation status of the Col II promoter and enhancer sequences within the gene's CpG islands and known to be critical for transcriptional regulation (Figure 2).

PCR amplifications were performed as described for PCR following restriction enzyme digestion. Two microliters of bisulfite converted DNA were used as template. The protocol for the PCR reaction was as follows: 95°C denaturation for 5 minutes, 45 cycles of denaturation at 95°C for 30 s, annealing at 55-60°C for 30 s, extension at 72°C for 20 s, followed by 1 cycle at 72°C for 10 min and 4°C indefinitely. The PCR products were separated by electrophoresis using a 2 % agarose TAE gel. Primer sequences and annealing temperatures are listed in Table 4. PCR products were cloned using TOPO PCR II TA cloning kit (Invitrogen), as per manufacturer's instructions. Six clones from each target site were sent to be sequenced by the University of Illinois Core Sequencing Facility.

### **p21 and p53 transfection studies**

Two to four million cells were cultured as non-adherent aggregates using a defined serum-free culture medium (Opti-MEM, Gibco). Twenty four-well (2 cm<sup>2</sup> wells) hydrogel-coated, ultra low attachment culture plates (Corning Costar, Pittsburgh, PA) were seeded with 1 million cell per well for the experiment that had 2 million cells per

treatment, whereas six-well (10 cm<sup>2</sup> wells) were used for the experiment that had 4 million cells per treatment. Cell culture conditions were as described above. After 24 hours of seeding, the chondrocytes were treated or not with 10 µM of 5-aza (Sigma-Aldrich) for 4 days, or transfected with adenoviral vector expressing p21, p53 or LacZ (control) cDNA at three different concentrations (2, 10 or 25 infectious particles per cell). Infection was performed in 1 mL of media (10 cm<sup>2</sup> wells) over four hours with occasional shaking. After four hours, 5 mL of fresh media was added and the samples were collected on day 4 as previously described. For the experiment that used 2 cm<sup>2</sup> wells, transfection was performed in 200 µL of media and 1.3 mL was added after the 4 hour incubation with occasional shaking. After pelleting, the media were aspirated from the samples and the pelleted aggregates were snap frozen in liquid nitrogen and stored at -80°C until processed. The adenoviral vectors were purchased from The University of Michigan's Vector Core laboratory. Collagen type II and EF1-α gene expression was assessed by real time PCR as described above.

### **Statistical analysis**

Statistical analyses were carried out using GraphPad Prism version 3.0 software. Comparisons between groups were made by two-way ANOVA. Dunnet's post-hoc test was applied when appropriate to assess the significance of differences between controls and experimental groups within each experiment. For all analyses, a p value of < 0.05 was considered to be significant.

## RESULTS

### Effects of 5-azacytidine on chondrocyte matrix gene expression

In non-adherent aggregate culture conditions, collagen type II expression by articular chondrocytes treated with 5-aza for 4 days was significantly greater ( $p=0.0049$ ) than control expression, as demonstrated by quantitative PCR and Northern Blot analyses (Figure 3). Further, 5-aza administration maintained collagen type II expression in monolayer culture; conditions that normally lead to phenotypic de-differentiation and loss of collagen type II expression<sup>9,68</sup> (Figure 4).

To investigate whether the effects of 5-aza were specific to collagen type II or affected the expression of other cartilage matrix genes, we performed quantitative PCR analyses of several other extracellular matrix genes. Collagen type I is not normally expressed by articular chondrocytes, and was therefore it was used as controls. Collagen type X is usually expressed by hypertrophic chondrocytes but not by articular chondrocytes; therefore, Col X expression was assessed to investigate whether 5-aza alters the chondrocytic phenotype as previously described<sup>57, 58, 66</sup>.

5-aza significantly up-regulated the expression of aggrecan, collagen types IX and XI, and link protein (Figure 5). Other extracellular matrix genes, such as collagen type VI and cartilage oligomeric matrix protein (COMP), were not affected, indicating that the upregulation of chondro-specific genes was not the consequence of a general effect on gene expression by 5-aza. Of note, collagen type I and type X were not affected, indicating that articular chondrocytes treated with 5-aza did not undergo de-differentiation or hypertrophy.

## **CpG methylation status of the collagen type II promoter and enhancer in human cell lines**

Given that the collagen type II gene promoter and chondrocyte-specific Sox-9-responsive enhancer are enclosed in CpG islands, and DNA methylation of CpG islands can impact gene expression, we investigated whether expression of collagen type II is associated with changes in the methylation status of these sequences. 5-aza is known to be incorporated into DNA as a cytidine analog during DNA replication. It covalently binds Dnmt1<sup>70, 71</sup> sequestering the enzyme and leading to a global reduction in genomic methylation<sup>72, 73</sup>.

In the first series of experiments, the methylation profiles of the collagen type II promoter and Sox 9 enhancer were assessed in chondrogenic (TC28a4 and HTB94), osteogenic (SAOS-2 and U2OS) and a generic (HeLa) cell lines. The methylation status of the 4,686 bases encompassing the promoter and enhancer CpG islands was analyzed by methylation-sensitive restriction digestion followed by PCR amplification (Figure 6). The methylation status of collagen type II varied among different cell lines and was specific to each cell line rather than showing phenotypic consistency or reflecting the expression status of collagen type II.

## **CpG methylation status of the collagen type II promoter and enhancer in primary human chondrocytes and osteoblasts**

It has been recognized that the epigenetic profiles of cell lines do not necessarily reflect the epigenetic profiles of primary cell types from which they are derived<sup>74</sup>. Therefore, we compared the methylation profiles of the Col II promoter and enhancer in

four paired articular cartilage and bone samples, collected from the same donors. The effect of monolayer de-differentiation on the methylation status of the Col II promoter/enhancer was also assessed by comparing passage 1 and passage 12 samples.

The Col II promoter/enhancer CpG islands contained very little methylation in either cell type and methylation patterns were not consistent within the cell types. Further, multi-passage monolayer de-differentiation did not alter the Col II methylation profile (Figure 7). In summary, the Col II promoter/enhancer methylation status did not reflect collagen type II expression in these ‘primary cell’ experiments.

### **The effect of 5-aza on collagen type II promoter and enhancer CpG methylation**

To determine whether 5-aza administration was affecting the CpG methylation status of the Col II promoter/enhancer CpG islands, methylation-sensitive restriction digestion, followed by PCR amplification, was applied to DNA samples from three ‘control’ and ‘5-aza treated’ samples where transcriptional up-regulation of collagen type II was previously confirmed (as in Figure 3). HeLa cells were also included as a generic control for these experiments. The result of these experiments demonstrated that Col II up-regulation after 5-aza treatment was not associated with altered methylation of the collagen type II promoter/enhancer sequences (Figure 8). In fact, no CpG demethylation was apparent in any sample.

## **Comprehensive analysis of collagen type II promoter and enhancer CpG methylation by bisulfite conversion and sequencing**

Restriction enzyme-based analyses of CpG methylation status are constrained to CpG sites within appropriate cleavage sites. To overcome these limitations, the methylation status of the collagen type II promoter and enhancer CpG islands in articular cartilage samples from six human donors were analyzed by bisulfite conversion and sequencing since this technique evaluates every CpG within the targeted sequence. Sixty-eight CpG sites in the promoter island and 17 CpG sites in the first intronic Sox-9-responsive enhancer were assessed by bisulfite conversion.

The bisulfite conversion results supported the previous findings that the collagen type II promoter and enhancer contain very few methylated CpGs and these methylation sites varied between individuals. The bisulfite conversion results from the enhancer analyses are presented in Figure 9. The promoter CpG island analyses were similar in the sporadic and infrequent distribution of methylated CpG sites.

## **Potential transcription factor targets of 5-aza**

Several transcription factors are known to regulate chondro-specific extracellular matrix gene expression and, more generally, the chondrocytic phenotype. As examples, during chondrogenesis, Sox-9, L-Sox-5 and Sox-6 control the expression of several chondrocyte-specific genes such as Col II, aggrecan, Col XI and link protein<sup>14-22</sup> In contrast, Snail and Slug repress expression of both Col II and aggrecan<sup>75</sup>. Therefore a gene candidate approach was used to investigate the expression of transcription factors known to influence expression of Col II in articular chondrocytes following 5-aza

treatment. The expression of transcription activator Sox-9 and p63, which drive expression of chondrocytic genes, were not up-regulated by 5-aza treatment. Further, the expression of several transcriptional repressors known to inhibit expression of the chondrocytic phenotype, such as twisted gastrulation, slug, and Ids 1-4 were not down-regulated by 5-aza (Figure 10). It does not appear that 5-aza alters Col II expression by influencing expression of transcription factors that regulate transcription of the collagen type II gene.

### **Potential growth factor targets of 5-aza**

Several growth and differentiation factors are known to support the chondrocytic phenotype and increase the expression of cartilage matrix genes; in particular, members of the TGF- $\beta$  and BMP ligand superfamily. Increased expression of one or more of these factors could account for the increased expression of Col II and other matrix genes, following 5-aza exposure (Figure 5).

Quantitative PCR analyses of growth factor expression after 5-aza administration showed that TGF- $\beta$ 3 and its downstream effector, connective tissue growth factor (CTGF), were significantly upregulated by 5-aza (Figure 11). However, we were not able to analyze the TGF- $\beta$ 3 promoter region to verify whether this upregulation correlated with altered methylation of the promoter region.

### **Potential collagen type II up-regulation by p53/p21 activation**

5-aza also induces cellular responses following genomic incorporation, through activation of the DNA damage response. This response leads to p53 activation,

p21Waf1/Cip1 up-regulation and cell cycle arrest<sup>76,77</sup>. Initial experiments in equine chondrocytes indicated that 5-aza does up-regulate p21 (Figure 12 upper panel), although the response of human OA chondrocytes was less consistent (data not shown). Hwang and colleagues<sup>78</sup> demonstrated CDK6/cyclin D1/p21 complexes regulate type II collagen expression in articular chondrocytes and proliferative stimulation of articular chondrocytes in monolayer cultures compromises collagen type II expression. Therefore we investigated whether direct over-expression of p53 and p21, mimicking the downstream consequences of the DNA damage response were sufficient to mimic 5-aza effects in articular chondrocytes.

In two independent experiments, p53 or p21 over-expression did not affect Col II expression by articular chondrocytes. Quantitative data from a Northern blot analysis are shown in Figure 12 (lower panel).

## **DISCUSSION**

In this study we investigated the effects of the DNA methylation inhibitor, 5-azacytidine, on the articular chondrocytic phenotype, with a specific focus on expression of the major extracellular matrix protein, collagen type II. Gene expression analyses demonstrated a significant increase in Col II expression following 5-aza exposure. Since collagen type II gene promoter and tissue-specific enhancer are encompassed in large CpG islands, we investigated whether the up-regulation of collagen type II expression observed in 5-aza treated chondrocytes was associated with changes in the methylation status of the promoter and/or enhancer sequences.



Initial analyses using methylation-sensitive restriction digestion followed by PCR amplification showed that the promoter and enhancer sequences contained very few methylated CpGs. This assay is an excellent screening assay since it is relatively inexpensive and rapid in comparison to other methods such as bisulfite conversion and sequencing. However, restriction-based techniques only evaluate CpGs that are contained within sites recognized by methylation-sensitive restriction enzymes. Some reports suggest that methylation changes in single CpG dinucleotides can activate expression of certain genes. Roach and co-workers have mapped the methylation status of MMPs-3, -9, and 13 and ADAMTS-4 promoters in human OA chondrocytes by methylation-sensitive restriction enzyme digestion and PCR. In all four enzyme sequences, the overall demethylation percentage was increased and some sites were preferably demethylated in OA samples<sup>62</sup>. Similar results were obtained in subsequent gene-targeted analyses<sup>63, 64</sup>. Similarly, Zimmermann *et al*<sup>79</sup> identified two adjacent CpGs that are differentially methylated in stem cells undergoing chondrogenesis, although the functional significance of this finding has not yet been established.

Methylation-sensitive restriction analyses of the Col II promoter and enhancer did not identify any significant CpG methylation patterns that reflected the chondrocytic phenotype, differential collagen type II expression or 5-aza-mediated demethylation. Therefore, we performed bisulfite conversion and sequencing of the collagen type II promoter and enhancer to comprehensively assess CpG methylation across these regions. The bisulfite results confirmed the findings obtained by methylation-sensitive restriction analysis. Collectively, the analyses revealed that the collagen type II promoter and enhancer are predominantly unmethylated, and sporadic methylation sites are not

consistently located across individual samples. These results indicate that Col II expression is not directly regulated by DNA methylation. This finding agrees with the current understanding that most strong CpG islands remain unmethylated, independent of their expression status. The majority of tissue-specific CpG islands that are differentially methylated are associated with genes that are important during development. Recent genome-wide analyses have indicated that tissue-specific differential methylation that correlates with gene expression is more likely to be located within CpG island shores, rather than within the CpG islands themselves <sup>74, 80, 81</sup>.

Since 5-aza did not seem to directly upregulate collagen type II through direct demethylation of the promoter or enhancer, we investigated whether 5-aza also induced expression of other cartilage matrix genes. 5-aza up-regulated the expression of several other chondro-specific extracellular matrix genes; aggrecan, collagen types IX and XI, and link protein. Collagen type X, exclusively expressed by hypertrophic chondrocytes, was not upregulated, indicating that these cells did not undergo hypertrophy following 5-aza treatment as previously suggested <sup>57, 58, 66</sup>. It has already been established that the aggrecan promoter is not differentially methylated in articular chondrocytes <sup>82</sup>, suggesting that these matrix genes are not individual targets of methylation-mediated regulation.

Several transcription factors are known to regulate chondro-specific extracellular matrix genes. For instance, during chondrogenesis, transcription factors Sox-9, L-Sox-5 and Sox-6 control the expression of several chondrocyte-specific genes such as Col II, aggrecan, Col XI and link protein <sup>14-22</sup>. Also, Snail, Slug and Ids are transcription factors that have the ability to repress expression of the chondro-specific genes, Col II and Agg

<sup>75</sup>. Therefore a gene candidate approach was used to investigate the expression of some of these regulatory factors in articular chondrocytes following 5-aza treatment. None of the transcription factors studied showed any changes that would explain 5-aza effects on upregulation of multiple extracellular matrix chondro-specific genes. Therefore, we investigated whether 5-aza upregulated growth factors responsible for chondrogenesis and maintenance of the chondrocytic phenotype <sup>4-7, 9, 68, 83-85</sup>. Quantitative PCR showed that TGF- $\beta$ 3 and its downstream regulator CTGF were significantly upregulated by 5-aza. Several studies indicate that TGF- $\beta$  signaling is critical for joint formation and maintenance of articular cartilage <sup>4-6, 85</sup>. TGF- $\beta$  signaling is not only important for maintenance of healthy articular cartilage, but also has chondro-protective activities in OA, by counteracting the effects of inflammatory cytokines <sup>86-89</sup>. Also, TGF- $\beta$ s stimulate expression of tissue inhibitors of metalloproteases (TIMPs), and increase collagen type II and proteoglycan synthesis by osteoarthritic chondrocytes <sup>90-93</sup>.

The relative contributions of donor age and cartilage pathology to our results cannot be clearly distinguished since all donors were "elderly" and even relatively "normal" cartilage collected from the TJA specimens was undoubtedly influenced by the severe and chronic pathology present throughout the involved joints. However, there is a considerable body of evidence that aging *per se* plays a role in the dysregulation of growth factor signaling, leading to cartilage degradation and chondrocytic phenotype alterations <sup>6, 93, 94</sup>. TGF- $\beta$ 2, TGF- $\beta$ 3 and TGF- $\beta$  receptor expression is decreased in old mice <sup>6</sup>. Corroborating this finding, TGF- $\beta$  signaling deficiency, through targeted disruption of Smad3 <sup>5</sup> or over-expression of a truncated, non-functional Tgfr2 <sup>4</sup> leads to articular cartilage changes that resemble osteoarthritis. Therefore, it is possible that TGF-

$\beta 3$  down-regulation by CpG methylation contributed to OA and the eventual need for joint replacement, and that 5-aza administration to OA chondrocytes relieved the methylation suppression of TGF- $\beta 3$  gene, leading to increased expression of chondrocytic matrix genes observed in our study. At this juncture, TGF- $\beta 3$  is a potential candidate for mediating the up-regulation of Col II and other cartilage matrix genes by 5-aza. Additional studies will need to be completed to clarify this possibility. Without question, the advanced age of the donor population (60-90 years of age) provide ample time for cumulative stochastic methylation events to influence the expression of critical homeostatic genes in articular cartilage and other organs.

The recent development of high throughput technologies (ChIP-chip and ChIP sequencing) <sup>95, 96</sup> have allowed researchers to identify and map epigenetic marks across the entire genome, to compare epigenetic features of specific regions between different tissues, and to associate epigenetic alterations with gene transcriptional states. These studies have greatly increased our understanding of DNA methylation profiles and associated effects on transcriptional activity. Most strong CpG islands promoters, such as the Col II island, are unmethylated independent of their gene expression profile; promoter hypomethylation and gene body methylation positively correlates with gene expression; promoters with few CpGs can be highly methylated and transcriptionally active; methylation of CpG island shores (regions of lower CpG density near (~2 kb) CpG islands) of promoter-associated CpG islands correlates with transcriptional inactivation; most tissue-specific DNA methylation is found in CpG island shores, rather than in CpG islands. Therefore it is unlikely the simple analyses of gene promoters using a gene

candidate approach will lead to the identification of regulatory regions in the genome that are influenced by CpG methylation and consequently responsive to 5-aza administration.

5-aza exerts its effects on genomic methylation predominantly by functioning as a cytidine analog during DNA replication. However, there are other mechanisms by which 5-aza might influence chondrocyte activity. The DNA/Dnmt adducts created by 5-aza incorporation triggers a DNA damage response resulting in p53 activation, increased p21Waf1/Cip1 expression, and cell cycle inhibition <sup>76</sup>. Zhu *et al*, 2004 <sup>77</sup> used deletion analyses and site-directed mutagenesis of the p21 promoter to demonstrate that 5-aza-CdR induction of p21Waf1/Cip1 expression requires two p53 binding sites in the p21 promoter. Comet assays and bisulfite sequencing were used to demonstrate that 5-aza-CdR-induced p21Waf1/ Cip1 expression was dependent on DNA damage as opposed to DNA demethylation.

Others have also demonstrated that 5-aza-CdR has replication-independent effects <sup>97-100</sup>. Firstly, 5-aza-CdR is able to induce Dnmt1 degradation through the proteosomal pathway <sup>100</sup>. Secondly, Dnmt1 might control DNA methylation in a replication-independent manner since it binds chromatin throughout G2 and M phases <sup>99</sup>. Finally, 5-aza can be incorporated into RNA, resulting in the inhibition of RNA and protein synthesis independently of any effect on the genome <sup>97, 98</sup>.

In summary, 5-aza and its analogs can influence cellular systems through their ability to decrease global methylation in replication-dependent or –independent manners, through DNA damage response, or through inhibition of mRNA and protein synthesis. These alternative mechanisms are particularly pertinent to the actions of 5-aza in chondrocyte populations, since phenotypically stable chondrocyte culture models such as

the one used in these experiments (low/no serum-suspension/non-adherent) do not support sustained cell proliferation. Adenoviral over-expression studies indicated that p53 or p21 overexpression in articular chondrocytes was not sufficient to up-regulate Col II or Agg gene expression, suggesting that DNA damage was not the mechanism mediating Col II up-regulation in response to 5-aza.

Accepting the 'process of elimination' outcomes in this experimental series, our results strongly suggest that 5-aza positively influences the age- and/or OA-associated suppression of extracellular matrix genes critical for cartilage homeostasis. Identification of the pathway(s) by which 5-aza exerts this activity could lead to therapeutic strategies to counter the cartilage matrix degradation that is the central pathological process in OA.

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## FIGURES AND TABLES

Table 1. Primers utilized to generate Northern Blot probes

Gene	Sense primer	Annealing
size	Antisense primer	temperature
Collagen type II	5'CCGAGGCAACGATGGTCAGC (822)	60°C
(380 bp)	5'TGGGGCCTTGTTACCTTTGA (1201)	
Aggrecan	5'TAGTGGAGCTACCCCAGTGC (5952)	58°C
(671 bp)	5'GGCATATGACGTGTCCCTCT (6622)	
p21	5'CGTGAGCGATGGAACTT (211)	54°C
(310 bp)	5'CTGTCATGCTGGTCTGC (520)	
EF1 $\alpha$	5'AAAATGACCCACCAATGGAA (1051)	62°C
(210 bp)	5'GCAGCATCACCAGACTTCAA (1261)	

Table 2. Primers utilized in the qPCR reactions

Gene	Sense primer	Annealing
size	Antisense primer	temperature
Collagen type I	5'AGCCAGCAGATCGAGAACAT (3841)	62°C
(303 bp)	5'CGCCATACTCGAACTGGAAT (4144)	
Collagen type II	5'ATGACAATCTGGCTCCCAAC (4062)	62°C
(220 bp)	5'CTTCAGGGCAGTGTACGTGA (4282)	
Collagen type IV	5'ATCGACAACCTGAGGGACAG (310)	62°C
(193 bp)	5'TGATAGCGCAGTCGGTGTAG (502)	
Collagen type IX	5'GCAGGTGGCTTACAAGTTGG (395)	62°C
(246 bp)	5'TGGAGACTTCCATCCAGTCC (640)	
Collagen type X	5'TGAGCAGCAACGTAAAAACG (2303)	62°C
(225 bp)	5'AGGAAATGCCGAGTTTCTCA (2527)	
Collagen type XI	5'TGAGCATGGTATTCAGCAAA (678)	62°C
(159 bp)	5'TTCTCCACGCTGATTGCTAC (836)	
Aggrecan	5'TGAGTCCTCAAGCCTCCTGT (6423)	62°C
(200 bp)	5'GGCATATGACGTGTCCCTCT (6622)	
Link Protein	5'TGTTTCTGAAGGGAGGCAGT (452)	62°C
(169 bp)	5'CCCAGTCGTGGAAAGTAAGG (620)	
COMP	5'GGAGATCGTGCAGACAATGA (1710)	62°C
(152 bp)	5'AGCTGGAGCTGTCCTGGTAG (1861)	
CTGF	5'AGTGGGAGGGTACCAGCAGA (1466)	62°C
(159 bp)	5'TTGATGGCTGGAGAATGCAC (1625)	

Table 2. (cont.)

Gene	Sense primer	Annealing
size	Antisense primer	temperature
TGF- $\beta$ 1	5'ACCTGAACCCGTGTTGCTCT (1299)	62°C
(225 bp)	5'AAGGCGAAAGCCCTCAATTT (1524)	
TGF- $\beta$ 2	5'CTGTCCCTGCTGCACTTTTG (856)	62°C
(172 bp)	5'GCAGGAGATGTGGGGTCTTC (1028)	
TGF-3	5'CAAATTCAAAGGCGTGGACA (997)	62°C
(242 bp)	5'CCCAGATCCTGTCGGAAGTC (1239)	
Sox-9	5'ATCAAGACGGAGCAGCTGAG (1561)	62°C
(161 bp)	5'GAGCTGGAGTTCTGGTGGTC (1721)	
IGF-1	5'CACTGCAGGAGGGACTCTGA (6693)	62°C
(151 bp)	5'GACATGGTATTTGGGGCCTTT (6843)	
p63	5'TCATGGATTTGGCACTAGCC (4353)	62°C
(166 bp)	5'TTGAGCTGAGGCCACAAGAA (4518)	
BMP-2	5'CTTCAAAGGGGCTGATCTGG (2791)	62°C
(242 bp)	5'AACAGAGGGTGGGCAGAAAA (3032)	
BMP-4	5'AAGCGTAGCCCTAAGCATCA (954)	62°C
(187 bp)	5'TGGTTGAGTTGAGGTGGTCA (1140)	
BMP-6	5'TACCCTCCTCCCCCAAAAC (1997)	62°C
(233 bp)	5'TGTGCGTCCTGTGGACTTCT (2229)	
BMP-7	5'GCAGGCAAAACCTAGCAGGA (1634)	62°C
(190 bp)	5'TTCGCACAGACACCAATGTG (1823)	

Table 2. (cont.)

Gene	Sense primer	Annealing
size	Antisense primer	temperature
Twist	5'GGAGTCCGCAGTCTTACGAG (644)	62°C
(161 bp)	5'CCAGCTTGAGGGTCTGAATC (805)	
Slug	5'GAGCATACAGCCCCATCACT (811)	62°C
(330 bp)	5'GCAGTGAGGGCAAGAAAAAG (1141)	
Snail	5'TTTACCTTCCAGCAGCCCTA (155)	62°C
(414 bp)	5'CCAGGCTGAGGTATTCCTTG (569)	
ID-1	5'CGGATCTGAGGGAGAACAAG (656)	62°C
(211 bp)	5'CTGAGAAGCACCAAACGTGA (867)	
ID-2	5'CGTGAGGTCCGTTAGGAAAA (201)	62°C
(236 bp)	5'ATAGTGGGATGCGAGTCCAG (437)	
ID-3	5'ACTCAGCTTAGCCAGGTGGA (551)	62°C
(166 bp)	5'AAGCTCCTTTTGTCTGTTGGA (717)	
ID-4	5'ATGGGATGAGGAAATGCTTG (1882)	62°C
(223 bp)	5'TGGAGGAAGGAAAGCAGAAA (2105)	
EF1 $\alpha$	5'AAAATGACCCACCAATGGAA (1051)	62°C
(210 bp)	5'GCAGCATCACCAGACTTCAA (1261)	



Table 3. Primers utilized in the PCR reactions following restriction enzyme digestion

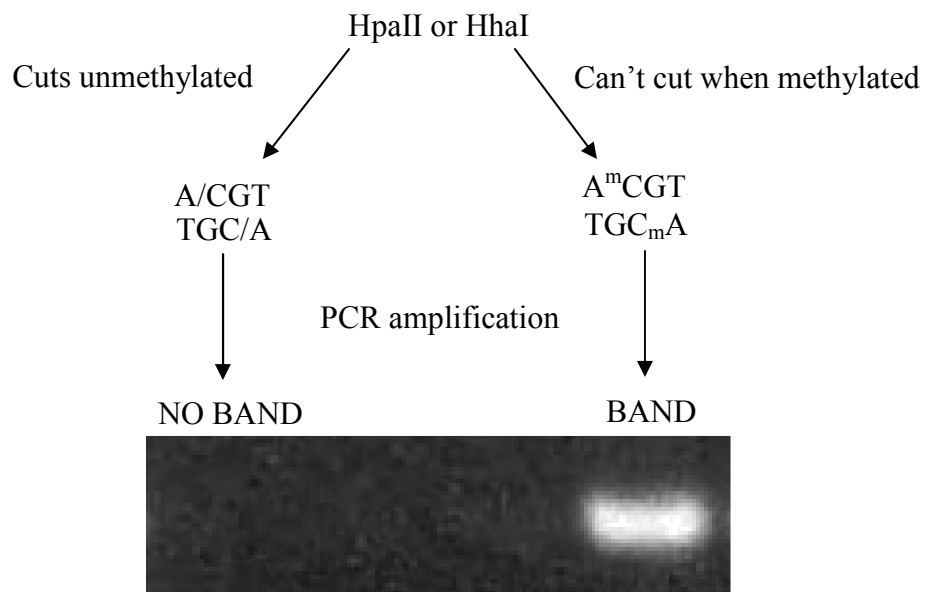
Gene	Sense primer	Annealing
size	Antisense primer	temperature
Collagen type II	5'CAAAAGGAAGGTCTGTTTGGGG (-1205)	65.5°C
(183 bp)	5'CAGAATTTCCCAGTCGGCC (-1022)	
Collagen type II	5'GGCCGACTGGGAAATTCTG (-1041)	66°C
(410 bp)	5'CTGCGTTGAAGGCTTCCTCT (-631)	
Collagen type II	5'ATGTCTTTTCCGTCCTTGGTCT (-383)	62°C
(318 bp)	5'GCTGGGCTGTAACCTGAACC (-65)	
Collagen type II	5'AGCGTGACTCCCAGAGAGG (-164)	62°C
(149 bp)	5'CTTTCGAGGCTGGCGAACT (-15)	
Collagen type II	5'AGTTCGCCAGCCTCGAAAG (-34)	64°C
(352 bp)	5'CTGAGGGACGCATGGAAAG (+318)	
Collagen type II	5'AGACGCTGGTGCTGCTGAC (+203)	66°C
(214 bp)	5'CTGCGTGTCTGTGCCTAAG (+417)	
Collagen type II	5'GGACACGCAGAAGTTCACCA (+407)	66°C
(429 bp)	5'TTCTTTGTGGCCTGGGATTT (+836)	
Collagen type II	5'AAATCCCAGGCCACAAAGAA (+816)	60°C
(419 bp)	5'CAACCGTAGCAATCGACCAG (+1235)	
Collagen type II	5'CTGGTCGATTGCTACGGTTG (+1215)	62°C
(515 bp)	5'TATTCCTCAAATGCCCAGGA (+1730)	
Collagen type II	5'CCTGGGCATTTGAGGAATACA (+1711)	62°C
(642 bp)	5'GTTTCAGCCCCATCTGGAAG (+2353)	

Table 3. (cont.)

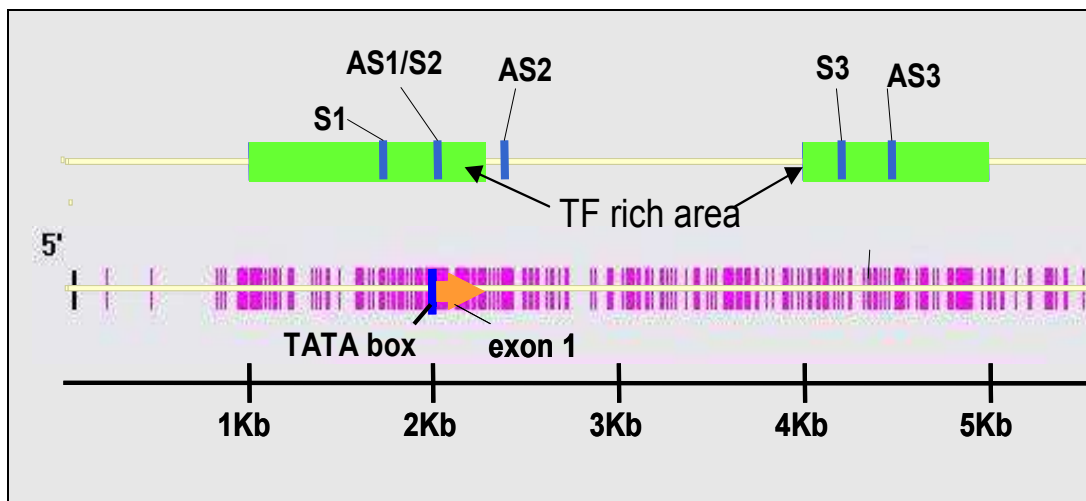
Gene	Sense primer	Annealing
size	Antisense primer	temperature
Collagen type II	5'CCCTCATCTGCATTTTCAGAGC (+2223)	62°C
(345 bp)	5'TTCGGGGAAGTGTGTTTGCTT (+2568)	
Collagen type II	5'GCAAAACAGTTCCCCGAAAG (+2550)	62°C
(169 bp)	5'CCTAGGTGTGGACGGAGGAG (+2719)	
Collagen type II	5'TCCTTTCTGGGGCAGTCATT (+2740)	62°C
(207 bp)	5'GGGGGAAATGCTAGGAGAGG (+2947)	
Collagen type II	5'CCTAGCATTTCCCCCTCTCC (+2932)	66°C
(549 bp)	5'TCCTCCAAGACTGCAGAGGAA (+3481)	
Cyclin D1	5'AGTAGCAGCGAGCAGCAGAGT (+68)	64°C
(296 bp)	5'ACCTCCTTCTGCACACATTTGA (+364)	
H19	5'CACTCATGGAAAAGCCTGAGAA (-1115)	62°C
(207 bp)	5'AGAGTTCACGGGTCCAATCAC (-908)	

Table 4. Primers utilized in the PCR reactions following bisulfite conversion

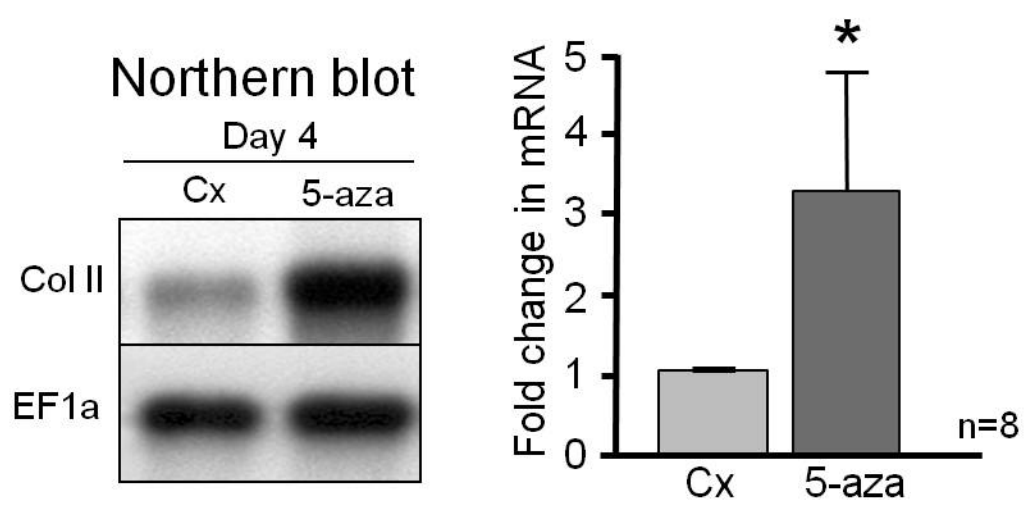
Gene	Sense primer	Annealing
size	Antisense primer	temperature
Col II	5'GTTTGGGGGGTAGGGGTTATTTTGT (-237)	60°C
331 bp)	5'CCCTCATACAAAAAACCTTAAAACAAAAA (+94)	
Col II	5'GTTTTAAGGGTTTTTTGTATGAGGG (-69)	60°C
(394 bp)	5'CCCTAAAACAAAATCCTTAATTAACAAAAC (+463)	
Col II	5'GTTTAGGGGAGAGATTTTAATTT (+2267)	55°C
(298 bp)	5'RAAAACTATTTTACTTCACC (+2565)	



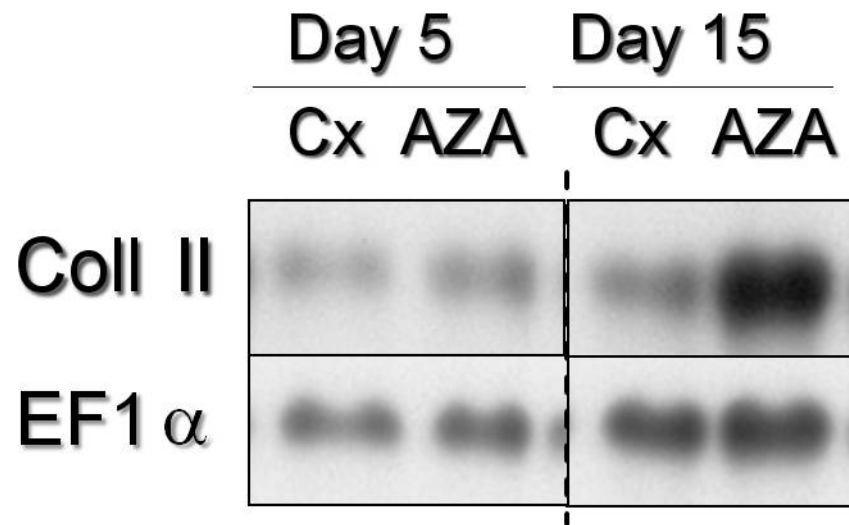
**Figure 1.** The effect of methylation on the outcome of digestion by the methylation-sensitive restriction enzymes HpaII and HhaI. In the absence of methylation, both enzymes cleave the template and no PCR amplicon is produced. Methylation impairs the restriction enzyme activity and a PCR product is generated from the intact DNA template.



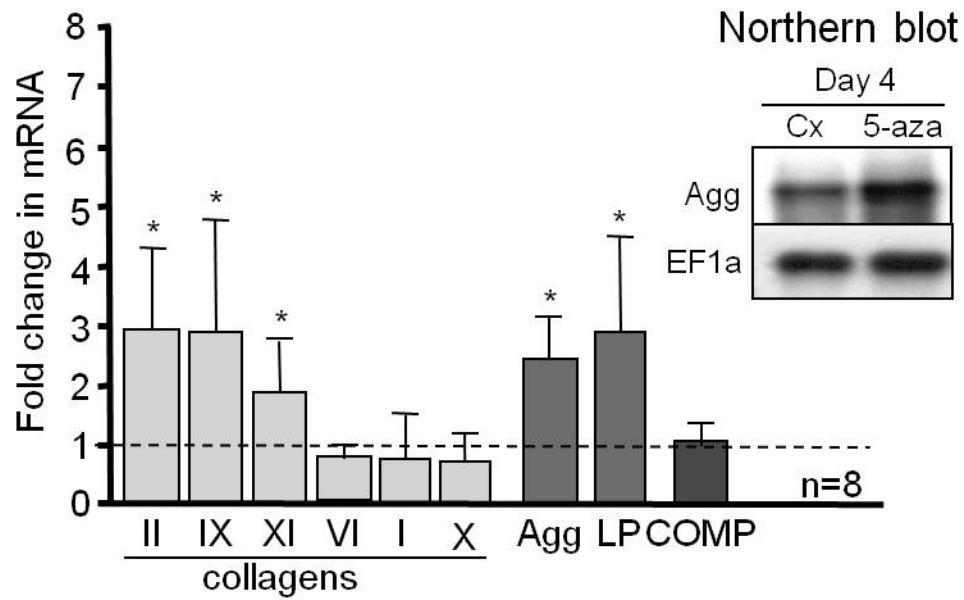
**Figure 2.** Representation of the collagen type II promoter region. Bisulfite converted primer pairs are labeled as S1-AS1, S2-AS2, S3-AS3. The transcription factors rich area is highlighted in green. The bottom scheme shows the TATA-box in blue, the exons in orange and the CpGs dinucleotides in pink.



**Figure 3.** Northern blot gel image and quantitative representation of 8 different experiments measuring collagen type II expression in chondrocytes treated or not with 5-aza for 4 days. 5-aza significantly increased Col II mRNA levels. Student's t-test  $p=0.0049$ .

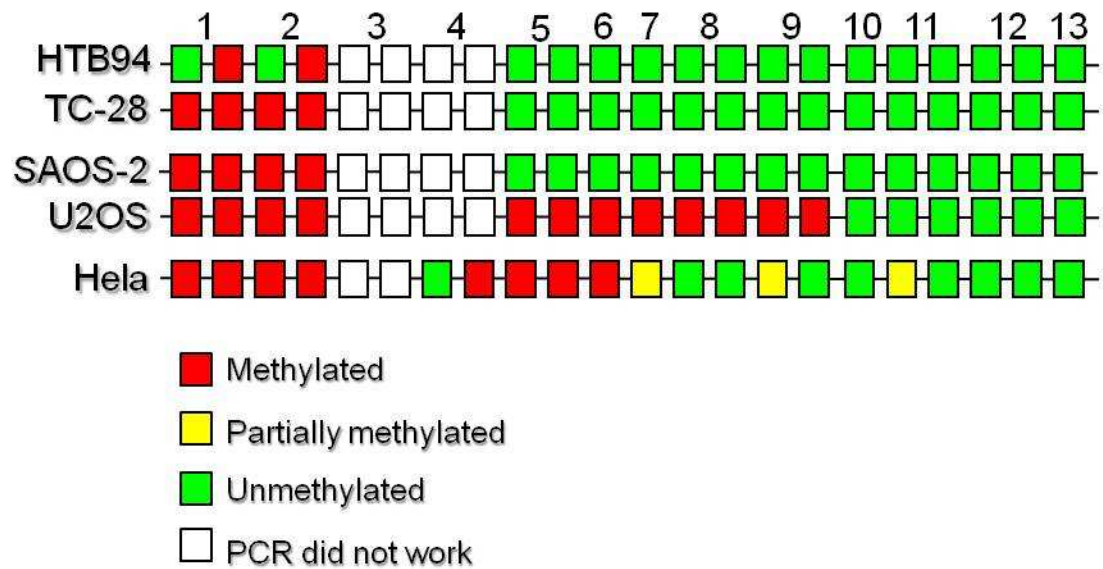


**Figure 4.** Northern Blot gel image of collagen type II expression in chondrocyte monolayer cultures treated or not with 5-aza for 15 days. 5-aza supported Col II mRNA expression in culture conditions known to induce phenotypic de-differentiation and loss of Col II expression, as in the control (Cx) group above.

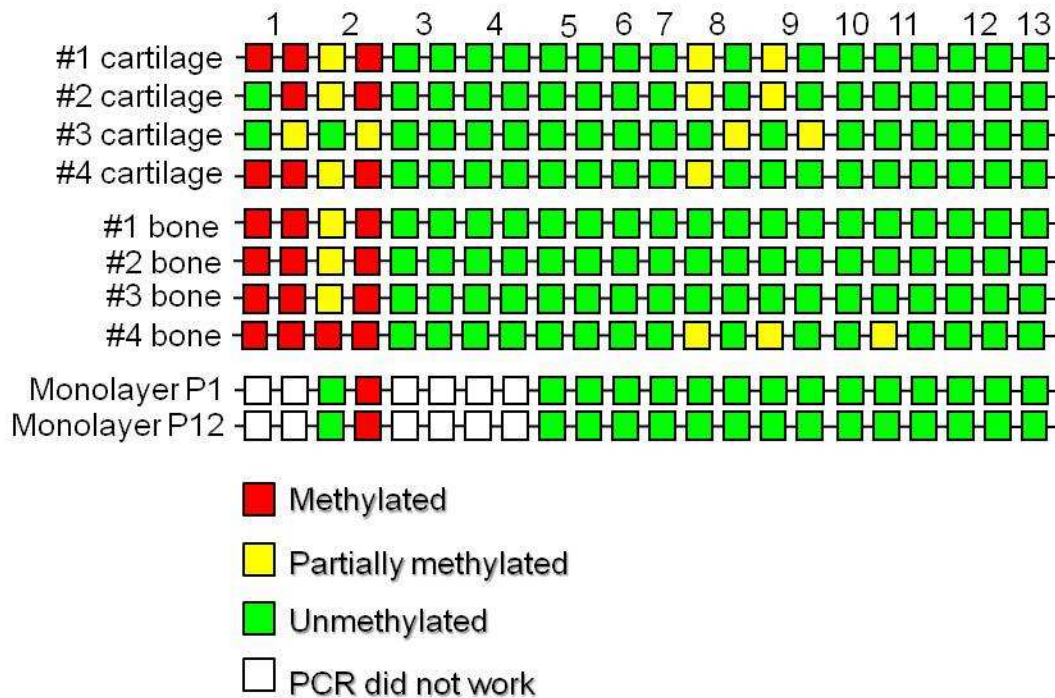


**Figure 5.** Northern blot of aggrecan expression in chondrocytes treated or not with 5-aza for 4 days (inset) and quantitative representation of 8 different experiments assessing the expression of collagen types II, IX, XI, VI, I, X, aggrecan (Agg), link protein (LP) and cartilage oligomeric matrix protein (COMP) expression in chondrocytes treated or not with 5-aza for 4 days. 5-aza significantly increased aggrecan ( $p= 0.0014$ ) and other chondro-specific extracellular matrix genes such as collagen type IX ( $p= 0.0091$ ) and XI ( $0.0183$ ), and link protein ( $p= 0.0403$ ).

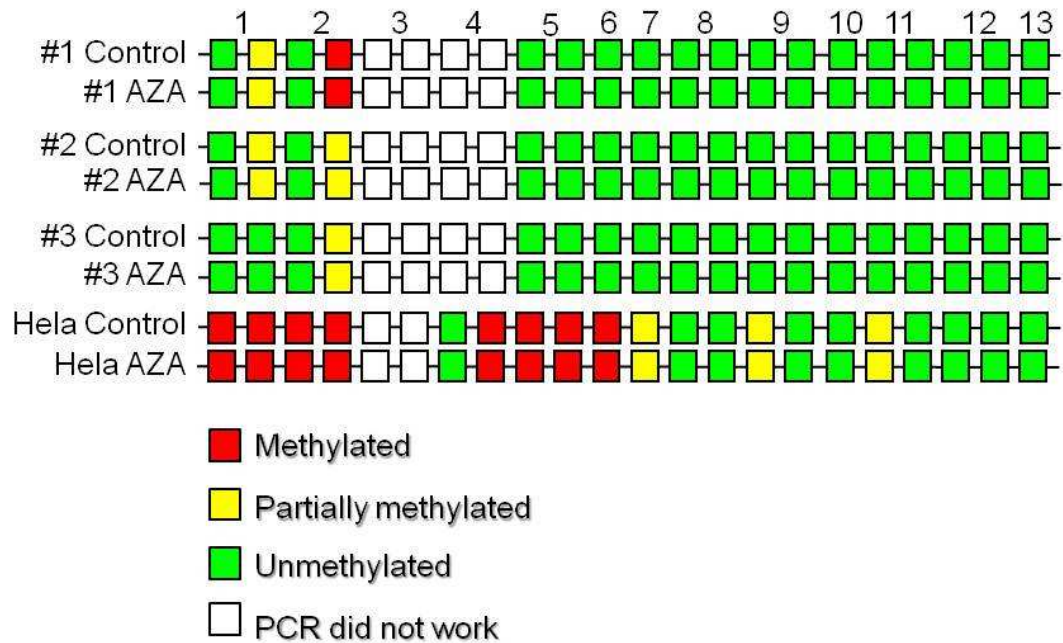




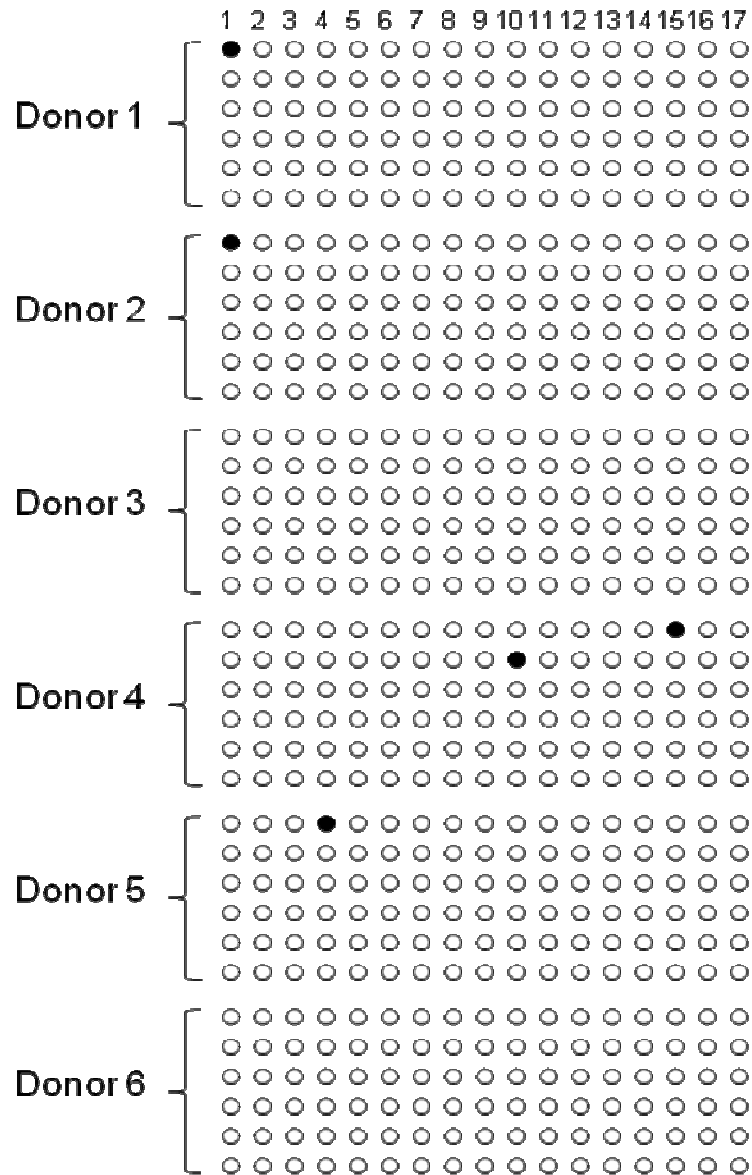
**Figure 6.** The methylation status of the collagen type II promoter by methylation-sensitive restriction enzyme digestion followed by PCR amplification in the chondrogenic cell lines HTB94, TC-28a4, osteogenic cell lines SAOS-2, U2OS and HeLa cells. The methylation status of the collagen type II promoter varies among different cell lines and the methylation patterns do not reflect phenotypic background or Col II expression.



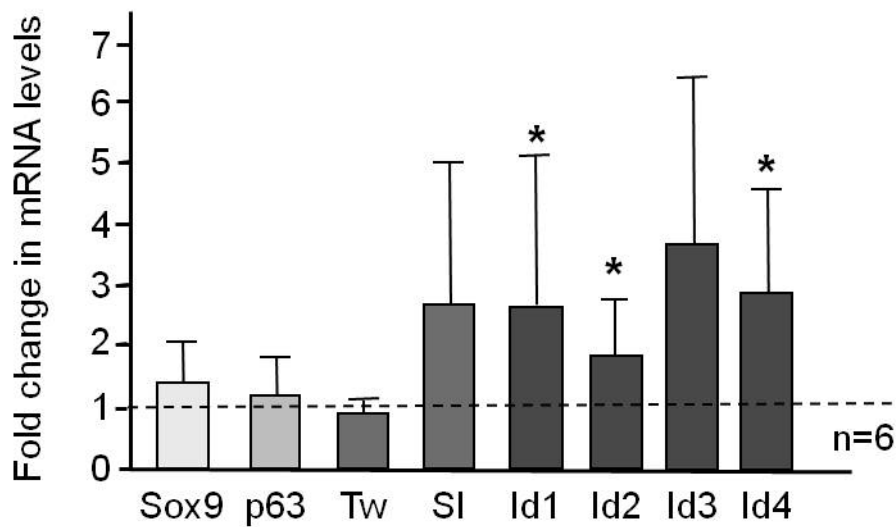
**Figure 7.** The methylation status of the collagen type II promoter in cartilage and bone from different human donors in P1 and P12 monolayer cultures. Methylation of CpG sites was identified by methylation-sensitive restriction enzyme digestion followed by PCR amplification. The methylation status of the collagen type II promoter varies within each tissue type and between the different tissues without consistent differences being apparent. The Col II promoter methylation status did not change during 12 monolayer passages, generating de-differentiated ‘chondrocytes’ that do not express collagen type II.



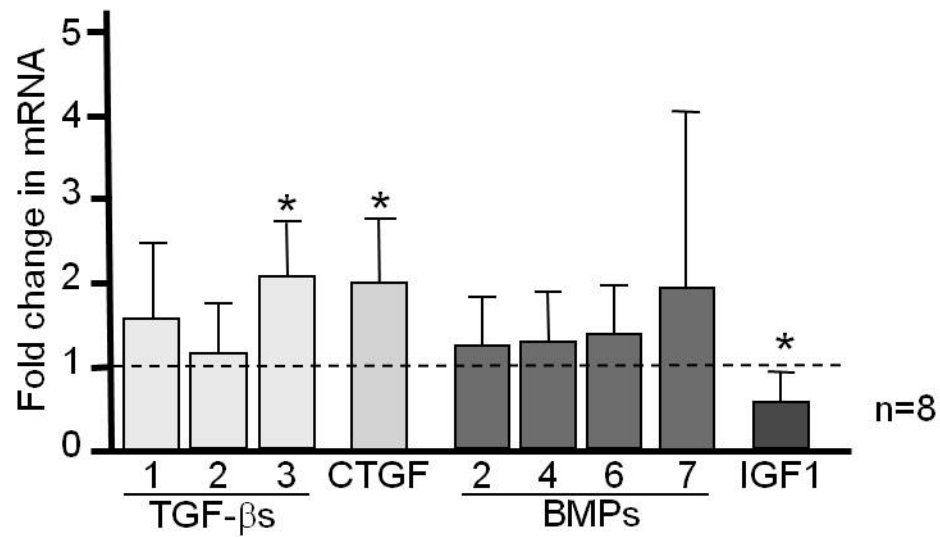
**Figure 8.** The methylation status of the collagen type II promoter in articular chondrocytes and HeLa cells treated with 5-aza for 4 days. The methylation status was determined by methylation sensitive restriction enzyme digestion followed by PCR amplification. The methylation profiles of the collagen type II promoter was not affected by 5-aza treatment.



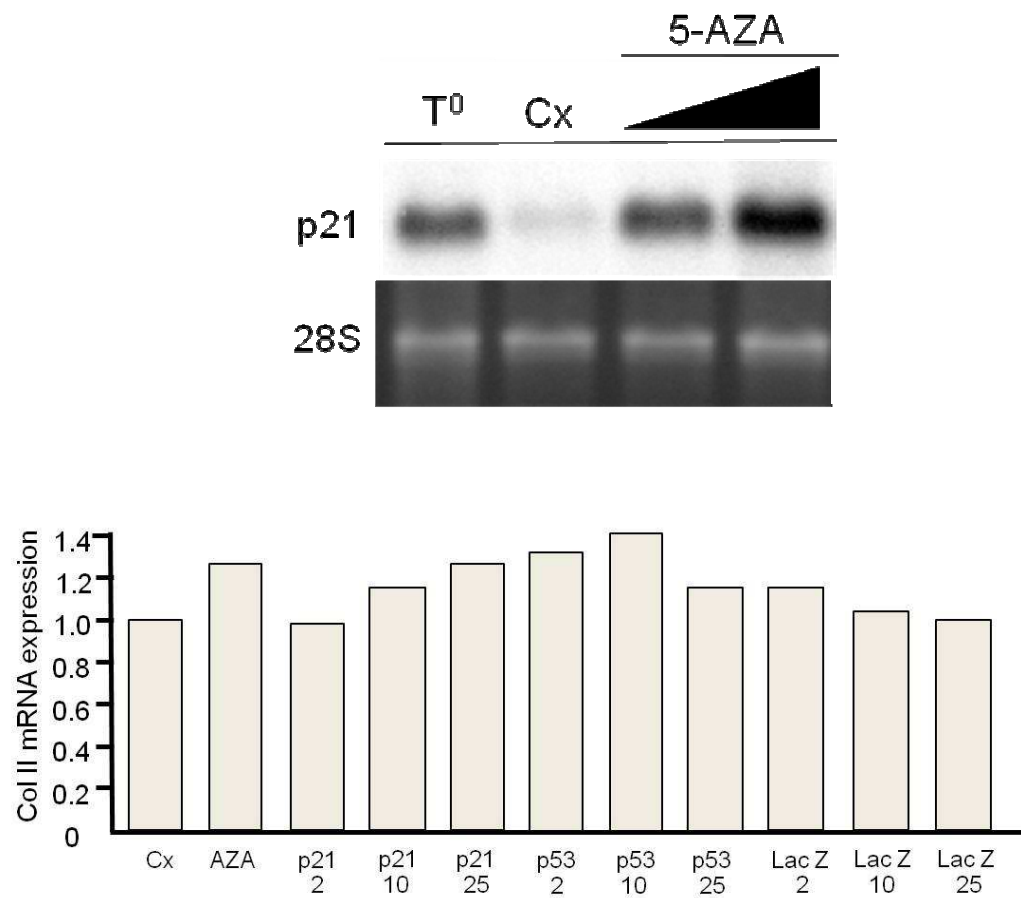
**Figure 9.** Identification of methylated CpGs in the Col II enhancer by bisulfite conversion followed by PCR. DNA from 6 different donors was used for bisulfite conversion. Six clones from each individual were sequenced. Open circles represent an unmethylated cytidine at a CpG site and dark circles represent a methylated cytidine at a CpG site. Methylated CpGs in the Sox-9 enhancer region are infrequent and sporadic, inconsistent with a gene regulated by methylation.



**Figure 10.** Effect of 5-aza administration on the expression of transcription factors that regulate the chondrocytic phenotype. Sox-9, p63, twisted gastrulation (TW), slug (Sl) and inhibitor of differentiation (Id) 1-4 in chondrocytes treated or not with 5-aza for 4 days. The expression of Sox-9 and p63, factors that trans-activate the Col II gene, were not up-regulated by 5-aza treatment. Further, the expression of transcriptional repressors such as twisted gastrulation, Slug, and Ids 1-4 were not affected by 5-aza. In fact, Ids 1, 2, and 4 were significantly upregulated ( $p = 0.0364$ ;  $0.0455$ ;  $0.0447$ , respectively).



**Figure 11.** Effect of 5-aza administration on the expression of growth factors known to support the chondrocytic phenotype. Expression of TGF-βs 1-3, connective tissue growth factor (CTGF), BMPs 2, 4, 6 and 7, and Insulin growth factor 1 (IGF1) in chondrocytes treated with 5-aza for 4 days was assessed by qPCR. Expression of TGF-β 3 ( $p= 0.0068$ ) and CTGF ( $p= 0.0073$ ) was significantly increased, whereas IGF1 expression was significantly down-regulated ( $p= 0.0102$ ).



**Figure 12. Upper panel.** 5aza dose-dependently stimulates p21 expression in equine articular chondrocytes. p21 expression was assessed by Northern blotting.

**Lower panel.** Collagen type II expression by human articular chondrocytes treated with 5-aza or infected with adenoviral plasmids expressing p53, p21 or Lac Z (2, 10 or 25 infectious particles per cell). Col II mRNA expression was assessed by Northern blotting. Adenoviral over-expression of p21 or p53 did not increase steady levels of Col II mRNA in articular chondrocytes.

## **CHAPTER 4: COLLAGEN TYPE X EXPRESSION AND 5' CpG METHYLATION STATUS IN OSTEOARTHRITIC ARTICULAR CHONDROCYTES**

### **INTRODUCTION**

Osteoarthritis (OA) is the most prevalent age-related disease in the world. The degeneration of articular cartilage that occurs in OA is progressive and irreversible; current treatments, with the exception of total joint arthroplasty, are predominantly palliative. OA affects over 60% of the US population over 65 years of age <sup>1</sup> and associated health costs amount to more than 60 billion dollars per year to the United States economy alone <sup>2,3</sup>. OA is characterized by progressive articular cartilage matrix depletion, fibrillation and loss <sup>4</sup>, deep matrix calcification <sup>5</sup>, and subchondral bone alterations <sup>6</sup>. Several pro-inflammatory cytokines involved in OA pathogenesis impact cartilage homeostasis <sup>2</sup>. In particular, interleukin (IL)-1 $\beta$ , IL-6 and soluble IL-6 receptor have been directly implicated in the down-regulation of collagen type II (Col II) during OA. These cytokines suppress Col II expression by increasing Sp3 occupancy in the promoter region of the Col II gene <sup>7,8</sup>. In addition, these cytokines stimulate the release of degradative enzymes such as matrix metalloproteases (MMP-2, -3, -8, -13), aggrecanases 1 and 2 (ADAMTS 4 and 5) and cathepsins, and suppress the synthesis of inhibitors such as the tissue inhibitors of metalloproteases (TIMPs). The change in matrix constitution and imbalance in synthesis: degradation ratio account for the cartilage destruction observed in OA <sup>4,9</sup>.



In addition to loss of extracellular matrix homeostasis, OA has also been linked to phenotypic alterations of the resident chondrocytes. Aberrant chondrocyte proliferation results in formation of cell clusters, or ‘chondrons’ and chondrocytes undergo phenotypic changes resembling the transitions that occur during hypertrophy. OA chondrocytes express collagens type I, IIA, III, and X, in addition to collagens IIB, VI, IX and XI that are usually expressed by normal chondrocytes<sup>10-12</sup>. These alterations have been demonstrated by comparative microarray analyses<sup>13</sup> and by differential immunostaining in OA and normal cartilage<sup>14</sup>. Therefore, dysregulation of mechanisms involved in articular chondrocyte phenotypic stability are implicated in the pathogenesis of osteoarthritis.

Epigenetics refers to “heritable changes in gene expression that occur without changes in DNA sequence”<sup>15</sup>. Epigenetics impact gene regulation through DNA methylation, histone protein modifications and chromatin organization. Overall, DNA methylation of CpG islands is associated with histone hypoacetylation and transcriptional repression. DNA methyltransferases incorporate a methyl residue to cytidines within CpG dinucleotide sequences. This modification can impair transcription by several means. Firstly, it can interfere with the recognition of DNA binding sites by transcription factors and other DNA binding proteins. Secondly, methylation can recruit CpG methyl-binding proteins impeding transcription factor binding. Lastly, methyl-CpG binding proteins can recruit histone deacetylases (HDACs), histone methyltransferases (HMTs) and chromatin-remodeling factors. These modifications condense chromatin structure, hiding potential binding sites for transcriptional activators and restricting occupancy. As a consequence, transcription is repressed<sup>16</sup>. However, promoter occupancy does not

depend solely in its epigenetic state. Transcriptional activation and repression are also regulated by competitive binding of promoter, enhancer and silencer elements <sup>7, 8, 17-20</sup>. Therefore, activation or repression of a gene is a collective consequence of the interplay of transcription factors and epigenetic constraints.

Epigenetic processes are involved in regulation of germline-specific genes, tissue-specific genes, imprinting, and aging <sup>21</sup>. A growing body of work indicates that epigenetics are also critical in a range of disease processes. Cancer cells are globally hypomethylated, although specific tumor suppressor genes are hypermethylated and transcriptionally suppressed <sup>22</sup>. Moreover, increased CpG methylation in CpG islands and concurrent loss of CpG methylation beyond island sequences is correlated with aging <sup>23</sup>. Environmental factors such as tobacco consumption, carcinogen exposure and diet *also* influence methylation profile and might predispose to certain diseases <sup>24</sup>. Several recent studies have demonstrated the importance of epigenetic gene regulation in chondrocyte development and homeostasis. HDAC4 functions as a negative regulator of chondrocyte hypertrophic differentiation by suppressing the activities of Runx2, a transcription factor required for hypertrophy <sup>25</sup>. HDAC activity is also necessary to support collagen type II expression in articular chondrocytes, and facilitate re-differentiation of (monolayer) de-differentiated chondrocytes <sup>26</sup>. The DNA methyltransferase inhibitor, 5-azacytidine (5-aza), induced expression of hypertrophic markers in phenotypically stable, chick articular chondrocytes <sup>27</sup>, suggesting that DNA methylation influences lineage commitment in chondrocyte populations. Cheung and colleagues <sup>28</sup> observed similar results when treating bovine fetal epiphyseal chondrocytes with 5-aza.

Epigenetics is also critical to the pathogenesis of OA. Young *et al.* (2005)<sup>29</sup> utilized a bovine nasal cartilage explant model to induce cartilage matrix degradation with IL-1 $\alpha$  and oncostatin M. Explants co-treated with HDAC inhibitors (trichostatin A or sodium butyrate) had decreased cartilage matrix degradation by blocking the induction of MMPs 1 and 13 and ADAMTS 4, 5 and 9 mRNA expression. Moreover, Roach *et al* (2005)<sup>30</sup>, linked osteoarthritic changes in articular chondrocyte to CpG methylation alterations in the promoter sequences of MMPs-3, 9, 13 and ADAMTS-4<sup>31</sup> that result in epigenetic de-silencing of these enzymes in OA. More recently, Zimmermann *et al.* (2008)<sup>32</sup> identified two CpG dinucleotides in the collagen type X promoter that were methylated in uncommitted mesenchymal stem cells but became de-methylated during *in vitro* chondrogenesis. The de-methylation of these CpGs correlated with collagen type X expression.

Given that osteoarthritic chondrocytes undergo phenotypic changes characteristic of hypertrophic chondrocytes and aberrantly express collagen type X, the objective of this study was to determine whether the methylation status of the CpG dinucleotide identified by Zimmermann *et al.* (2008)<sup>32</sup> in the context of stem cell chondrogenesis is also linked to collagen type X expression in osteoarthritic chondrocytes. The study addressed the hypothesis that induction of collagen type X expression in osteoarthritic articular chondrocytes is related to demethylation of cytidines positioned 1,680 and 1,674 bases 5' to the collagen type X start site.

## **METHODS**

### **Sample collection**

Human articular cartilage was harvested from 11 osteochondral tissue specimens retrieved from total joint arthroplasty (TJA) procedures obtained from Carle Clinic in Urbana, IL following total knee replacement procedures. Articular cartilage was obtained from 3 women (60-79 years) and from 8 men (59-86 years). In addition, a bone sample was collected from a female donor (90 years) for use as a control. This project was approved by both the University of Illinois Ethics Committee and the Carle Clinics Ethics Committee. Informed consent was obtained from the patients before surgery.

The articular cartilage specimens presented with variable degrees of arthritic change, both between and within specimens. Therefore, articular cartilage was categorized according to macroscopic characteristics; samples that were not fibrillated were considered 'normal', whereas cartilage samples with surface fissures or fibrillation were considered to be 'osteoarthritic'. Severely degenerative tissues and osteophytic cartilage were excluded. Cancellous bone was harvested from the central region of the proximal tibial fragment. The cartilage and bone were snap frozen in liquid nitrogen and stored at -80°C for until needed for RNA and DNA isolation.

### **RNA extraction**

The articular cartilage samples were pulverized under liquid nitrogen in a Spex 6700 Freezer/Mill (Spex Industries Inc., Metuchen, NJ, USA). Approximately 200 mg of pulverized cartilage was saved for DNA extraction and stored at -20°C. Total RNA was extracted from articular cartilage using previously described protocols<sup>33</sup>. Briefly, 2-3 g

of powdered cartilage was mixed with 8 mL of guanidinium isothiocyanate lysis buffer (4M Guanidinium thiocyanate (Fisher Scientific, Philadelphia, PA, USA), 100mM Tris-HCl (Fisher Scientific) and 25 mM EDTA (Fisher Scientific), 80  $\mu$ L of  $\beta$ -mercaptoethanol (Sigma-Aldrich, St. Louis, MO) and homogenized in a Ultra-Turrax T25 homogeneizer (Janke & Kunkel, IKA-Labortechnik, Staufen, Germany) for 2 minutes. The sample was placed on ice and 650  $\mu$ L of 25% Triton X-100 (Sigma-Aldrich Corporate) was added to the samples. After 10 minutes on ice, the sample was centrifuged at 805 rcf for 5 minutes in a refrigerated centrifuge (Centrifuge 5810 R, Eppendorf, Westbury, NY). The supernatant was transferred to an ultracentrifuge tube (Beckman Coulter Inc.), 8 mL of 3 M sodium acetate was added and the sample was mixed. Thirteen milliliters of water-saturated phenol (Fisher Scientific) and 3 mL of chloroform: isoamyl alcohol (Fisher Scientific) (49:1) were added, the sample was well mixed and placed on ice for 10 minutes. The aqueous and organic phases were separated by centrifugation (48,394 rcf for 30 minutes) in an ultracentrifuge (Beckman Coulter Inc.). Phenol: chloroform (1:1) extraction was repeated until the insoluble debris separating aqueous and organic phases was minimal. The aqueous phase was transferred to an ultracentrifuge tube (Beckman Coulter Inc), 13 mL of isopropanol (Fisher Scientific) was added and the sample was kept at -20°C overnight to facilitate RNA precipitation. RNA was pelleted by centrifugation at 72,128 rcf for 30 minutes. The pellet was transferred to a 1.5 mL microcentrifuge tube (Fisher Scientific) and washed with 70% ethanol (PHARMCO\_AAPER, Shelbyville, KY) in diethyl pyrocarbonate (Sigma-Aldrich) (DEPC)-treated water. Proteoglycans co-precipitated with the RNA were removed using the RNeasy “clean up” protocol (Qiagen, Valencia, CA). Briefly, the

pellet was dissolved in 600  $\mu$ L of Lysis Buffer RLT and mixed with 900  $\mu$ L of 70% ethanol. The mixed solution was applied to the RNeasy column and spun at 8,100 rcf for 15 s (Denville 260D centrifuge – Denville Scientific Inc., Metuchen, NJ, USA). The column was rinsed with 400  $\mu$ L of Wash Buffer RW1, and 80  $\mu$ L of DNase I in RDD Buffer (Qiagen) was applied to the column. After 15 minutes at room temperature, the column was washed with 400  $\mu$ L of RW1, followed by two 500  $\mu$ L washes with Wash Buffer RPE. The column was dried by centrifugation at 8,100 rcf for 2 minutes and then the RNA was eluted with 55  $\mu$ L of RNase free water.

### **Reverse transcription reaction**

First strand cDNA was synthesized by reverse transcription using First Strand cDNA Superscript II (Invitrogen Corporation, Carlsbad, CA) and Oligo dT as per manufacturer's instructions. Briefly, one microgram of total RNA was brought up to 10  $\mu$ L and then combined with 1  $\mu$ L of oligo (dT) (0.5 $\mu$ g/ $\mu$ L) and 1  $\mu$ L of 10 mM dNTPs. The sample was incubated in a 65°C water bath for 5 minutes and then placed on ice for 1 minute to denature the RNA and allow binding of the oligonucleotides. The following reagents were added: 2  $\mu$ L 10x First Strand Buffer, 2  $\mu$ L 0.1M DTT, 2  $\mu$ L 50 mM MgCl<sub>2</sub> and 1  $\mu$ L RNase out. After a gentle mix and brief centrifugation, the sample was incubated at 42°C water bath for 2 minutes, 1  $\mu$ L (50 units) of SuperScript II Reverse Transcriptase was added. The sample was then incubated at 42°C for 50 minutes. The reaction was stopped by placing the sample in a 70°C water bath for 15 minutes. The RT reactions were diluted (1:10 ratio) with water and stored at -20°C until further use.

## Quantitative PCR

Quantitative PCR (qPCR) was used to assess collagens type II and type X, aggrecan and alkaline phosphatase gene expression profiles in the cartilage samples. The PCR reactions were carried out in a final volume of 25  $\mu$ L. The 2x SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) was used for these reactions. This reagent contains 100 nM KCl, 40 mM Tris-HCl, 0.4 mM of each dNTP, 50 units/mL of *Taq* polymerase, 6 mM  $MgCl_2$ , and 20 nM SYBR Green I. The SYBR Green Supermix (12.5  $\mu$ L) was added to 1  $\mu$ L of the sense (10 pMol) and 1  $\mu$ L of the antisense primers. Autoclaved distilled water (5.5  $\mu$ L) and 5  $\mu$ L of cDNA template were also added. The initial denaturation step was carried out at 95°C for 3 minutes, then 45 cycles of denaturation at 95°C for 10s, annealing temperature of 62°C for 30s and polymerase extension at 72°C for 20s followed. The samples were denatured at 95°C for 1 min before starting the melting curve protocol that consisted of decreasing the temperature at 55°C for 1 minute followed by increments of 0.5°C every 10 s till 95°C was reached. The primer combinations used in these experiments were designed for annealing at 60-62°C, however optimal annealing temperatures were determined by temperature gradient analyses spanning 54-64°C. The specificity of the PCR products was monitored by melting curve analyses, PCR fragment cloning and sequencing. Graphs showing the fluorescence intensity were plotted for each cycle. Quantitative results were accessed using the cycle threshold determined to represent the onset of the linear phase of amplification, as calculated by the thermal iCycler software (Bio-Rad). To assess potential contamination, water was used as negative control for each on the reactions

instead of cDNA. The qPCR data was normalized by the elongation factor-1 alpha (EF1 $\alpha$ ) expression. The primers are listed in Table 5.

### **DNA extraction**

Approximately 200 mg of powdered cartilage or bone was digested overnight at 50°C using 2 mL of proteinase K digestion buffer (100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 0.5% SDS and 0.1 mg/mL of proteinase K). The DNA was extracted in 1 mL of phenol and 1 mL of chloroform: isoamyl alcohol (24:1). After vortexing, the sample was centrifuged at 8,100 rcf for 10 minutes in a bench top centrifuge (Denville 260 D, Denville Scientific Inc., Metuchen, NJ) to separate the organic and aqueous phases. The aqueous supernatant was transferred to a new microcentrifuge tube and 500  $\mu$ L of 7.5 M ammonium acetate and 2 mL of 100% ethanol were added. The sample was mixed by inversion of the tube and allowed to precipitate at room temperature for 5 minutes before being spun at 8,100 rcf for 2 minutes. The supernatant was discarded and the pellet was washed with 70% ethanol, air dried and reconstituted in 270  $\mu$ L of TE buffer. The sample was re-precipitated overnight at -20°C using 30  $\mu$ L of 3 M Na acetate and 900  $\mu$ L of 100% ethanol. The sample was pelleted at 8,100 rcf for 10 minutes, washed in 70% ethanol, air dried, resuspended in 200  $\mu$ L of TE buffer and quantified by UV spectrophotometry. The sample was stored at -20°C until needed.



### **Methylation-sensitive restriction enzyme digestion**

As reported by Zimmermann *et al.* (2008)<sup>32</sup>, the cytidines located at position -1,680 and -1,674 upstream of the transcription start site of the collagen type X gene were >50% demethylated during chondrogenesis and the degree of methylation correlated with collagen type X expression during mesenchymal stem cell differentiation. These sites (ACGT) are recognized by the methylation-sensitive restriction enzyme HpyCH4IV. To determine whether collagen type X expression by human osteoarthritic chondrocytes correlates with demethylation of these cytidines, primers spanning these putative restriction sites were designed.

Sufficient DNA was able to be extracted from ten of the initial 15 human OA cartilage samples for CpG methylation analysis. Five hundred nanograms of genomic DNA extracted from articular cartilage, were digested or not (control) with HpyCH4IV (New England Biolabs, Ipswich, MA) in a 40 µL reaction at 37°C overnight. EcoRV (Invitrogen) was also added to both control- and HpyCH4IV-digested samples to reduce the fragment size of the genomic templates. Twenty units of both enzymes were added to the digestion buffers to ensure complete digestion of the genomic DNA. Primers spanning an HpyCH4IV cleavage site in the collagen type II promoter were used to assess complete DNA digestion, given that previous studies have shown that the collagen type II promoter is predominantly unmethylated in chondrocytes. The restriction digestion was stopped by heating the samples at 65°C for 25 minutes. The samples were diluted with 40 µL of water.

### **PCR amplification**

Five microliters of the diluted samples (31.25 ng DNA) were used as templates for PCR reactions. The PCR reaction (25  $\mu$ L) contained 2.5  $\mu$ L of 10x PCR buffer (Invitrogen), 1  $\mu$ L of 50 mM MgCl<sub>2</sub>, 0.5  $\mu$ L of 10 mM dNTPs (Invitrogen), 10 pMol of the sense primer, 10 pMol of the antisense primer and 0.3  $\mu$ L of *Taq* DNA polymerase (Invitrogen), in addition to the DNA template. The protocol for the PCR reaction was as follows: 95°C denaturation for 5 minutes, 25 cycles at 95°C for 30 s, 62°C for 30s, 72°C for 20s, 1 cycle at 72°C for 10 min and 4°C indefinitely. PCR products were separated by electrophoresis using a 2 % agarose TAE gel. The primers are listed in Table 6.

### **Band quantification**

The 2% agarose TAE gel was stained with ethidium bromide and photographed using a Kodak EDAS 290 system (Carestream Health, Inc., Rochester, NY) for band intensity comparison between control and samples digested with HpyCH4IV (Figure 13). Band intensity was quantitatively analyzed by using ImageJ software (free download available at <http://rsbweb.nih.gov/ij/>).

### **Statistical analysis**

Statistical analyses were carried out using GraphPad Prism version 3.0 software. Differences in the expression of collagen type X mRNA and in the extent of CpG demethylation between grossly normal and OA cartilage specimens were analyzed by Student's t- tests. Correlations between Collagen type X and ALP expression, and between demethylation and collagen type X expression and were determined by

Pearson's correlation coefficient. For all analyses, p values of < 0.05 were considered to be significant.

## RESULTS

There was a greater than 400-fold range in the levels of collagen type II mRNA expression although two samples were responsible for this considerable variation. Discounting specimens 3 and 4 (Figure 14), collagen type II expression showed approximately five-fold variation in the remaining 13 samples. Aggrecan mRNA expression showed similarly restricted variation across the samples. Interestingly, there were no obvious differences in collagen type II or aggrecan expression between the grossly 'normal' and 'OA' specimens.

Collagen type X expression in the articular cartilage samples showed over 600-fold variation between specimens (Figure 15), indicative of considerable phenotypic alterations in these cases. Collagen type X expression levels in grossly 'normal' and 'osteoarthritic (OA)' specimens were not significantly different (p value = 0.98). ALP expression was assessed as an independent marker of the hypertrophic phenotype. ALP expression was also highly variable and was correlated to Col X expression (Figure 16; p = 0.0343) in samples that presented higher Col X expression (greater than '100 fold' in Figure 15; Pearson  $r = 0.5886$ ;  $R^2 = 0.3464$ ).

Cleavage activity of the methyl-sensitive restriction enzyme HpyCH4IV was used to assess the methylation status of cytidines located at position -1,680 and -1,674 upstream of the transcription start site of the collagen type X gene. To test for complete DNA digestion by the enzyme HpyCH4IV, we used primers that spanned an HpyCH4IV

site in the collagen type II promoter (Figure 17). Previous studies demonstrated that this region of the collagen type II promoter is predominantly unmethylated in chondrocytes (see Chapter 3). As demonstrated in Figure 17, HpyCH41V digestion was complete in all cases. Demethylation of the targeted cytidines varied among individuals and among different samples from the same individual (Figure 18), but the level of demethylation, in general, was low among all the samples studied (between 4 and 12%). Of note, the extent of CpG demethylation in a single non-collagen type X-expressing bone specimen (8.49%) was comparable to the levels detected in the OA cartilage samples (Figure 18). There was no significant difference in ‘% CpG demethylation’ between grossly normal and OA specimens ( $p = 0.34$ ). Further, there was no correlation between % demethylation and collagen type X expression (Pearson  $r = -0.1452$ ;  $R^2 = 0.02107$ ; Figure 19). In essence, the targeted cytidines in the collagen type X promoter are highly methylated (greater than 88%) independent of collagen type X expression level.

## DISCUSSION

This study was conducted to determine whether collagen type X expression in osteoarthritic chondrocytes is related to demethylation of the cytidines at position -1,680 and -1,674 upstream of the transcription start site of the collagen type X gene. In the context of this investigation, we also assessed the expression of genes that characterize the chondrocyte phenotype (Collagen type II and aggrecan)<sup>4, 34</sup> and the expression of ALP; an independent marker of the hypertrophic phenotype<sup>35, 36</sup>. As expected, aggrecan and collagen type II mRNAs were detected in all specimens; however, there was a broader range of Col II expression among samples, probably reflecting differing degrees

of degenerative pathology<sup>37</sup>. Collagen type X mRNA expression varied considerably (approximately 600-fold variation) although in all OA specimens. ALP expression was correlated with collagen type X expression, consistent with several previous studies<sup>5, 14, 36, 38-40</sup> and indicating that these transcriptional alterations reflect a phenotypic change as opposed to an effect on a single gene.

Our results do not support the hypothesis that induction of collagen type X expression in osteoarthritic articular chondrocytes is related to demethylation of the -1,680 and -1,674 cytidines, as reported by Zimmermann *et al.* (2008). In the absence of an appropriate methylation-resistant isoschizomer as a control for complete digestion, we used primers spanning a region of the collagen type II promoter, which contains an HpyCH4IV restriction site and that we have identified as being unmethylated in chondrocytes, to control for complete digestion. In contrast to the results reported by Zimmermann *et al.*, where demethylation of the -1,680 and -1,674 cytidine bases exceeded 50% in collagen type X-expressing chondrogenic MSCs. In our study, there was highly variable expression of collagen type X by human articular chondrocytes, but very little demethylation of the targeted CpG sites. The lack of correlation in the current study might be a consequence of phenotypic heterogeneity in the chondrocyte populations under examination; a relatively small number of cells could be responsible for overall collagen type X expression yet make minimal contributions to the methylation analyses of the targeted CpG sites. Accepting this possibility, the in vitro chondrogenesis model used in Zimmermann *et al.*'s study also generates a phenotypically heterogeneous population within the three-dimensional pellet cultures, and so would also be subject to similar variations.

It is also possible that the regulation of collagen type X expression by differentiated chondrocytes differs from regulatory mechanisms in stem cells undergoing chondrogenesis and is not dependent on the methylation status of the targeted CpG sites. Self-evidently, a statistical correlation does not indicate any causal or functional association. Stem cells genomes are highly methylated, in comparison to those of differentiated cell populations, and that there is a decrease in overall methylation during differentiation <sup>41</sup>. Therefore, the demethylation of the CpG sites observed by Zimmerman and colleagues could reflect a non-specific genome-wide demethylation during chondrogenic differentiation. Further, the genomic sequence surrounding the targeted CpG sites do not correspond to a recognized transcription binding site and the targeted CpG pairs are not conserved in murine or equine genomes, arguing against an important functional activity. Transcriptional activation and repression are regulated by competitive binding of promoter, enhancer and silencer elements <sup>7, 8, 17-20</sup>, independently of any epigenetic influences.

In summary, collagen type X expression by human osteoarthritic chondrocytes is not linked to the methylation status of the of cytidines located at position -1,680 and -1,674 upstream of the transcription start site of the collagen type X gene.

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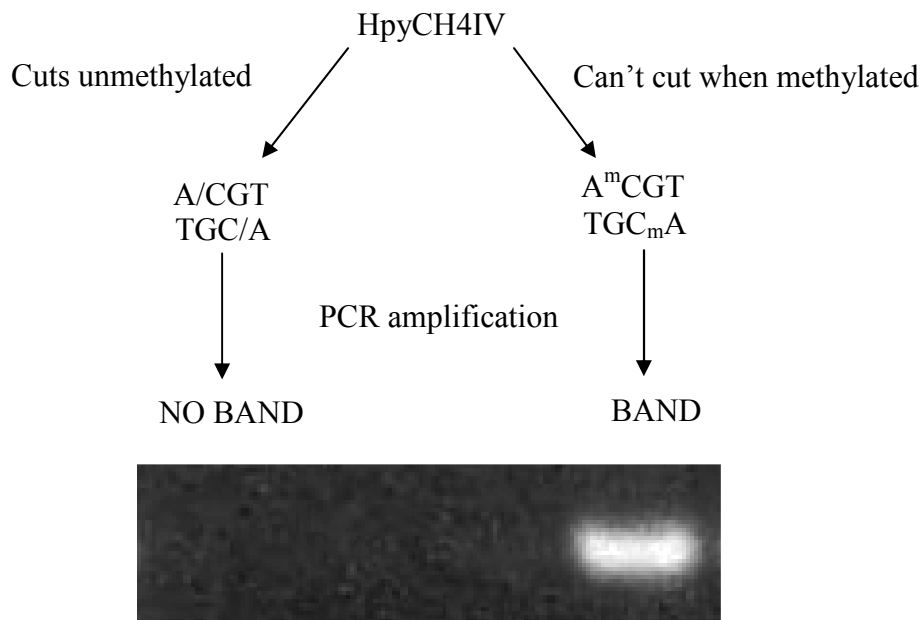
## TABLES AND FIGURES

Table 5. Gene specific primers utilized in the qPCR reactions.

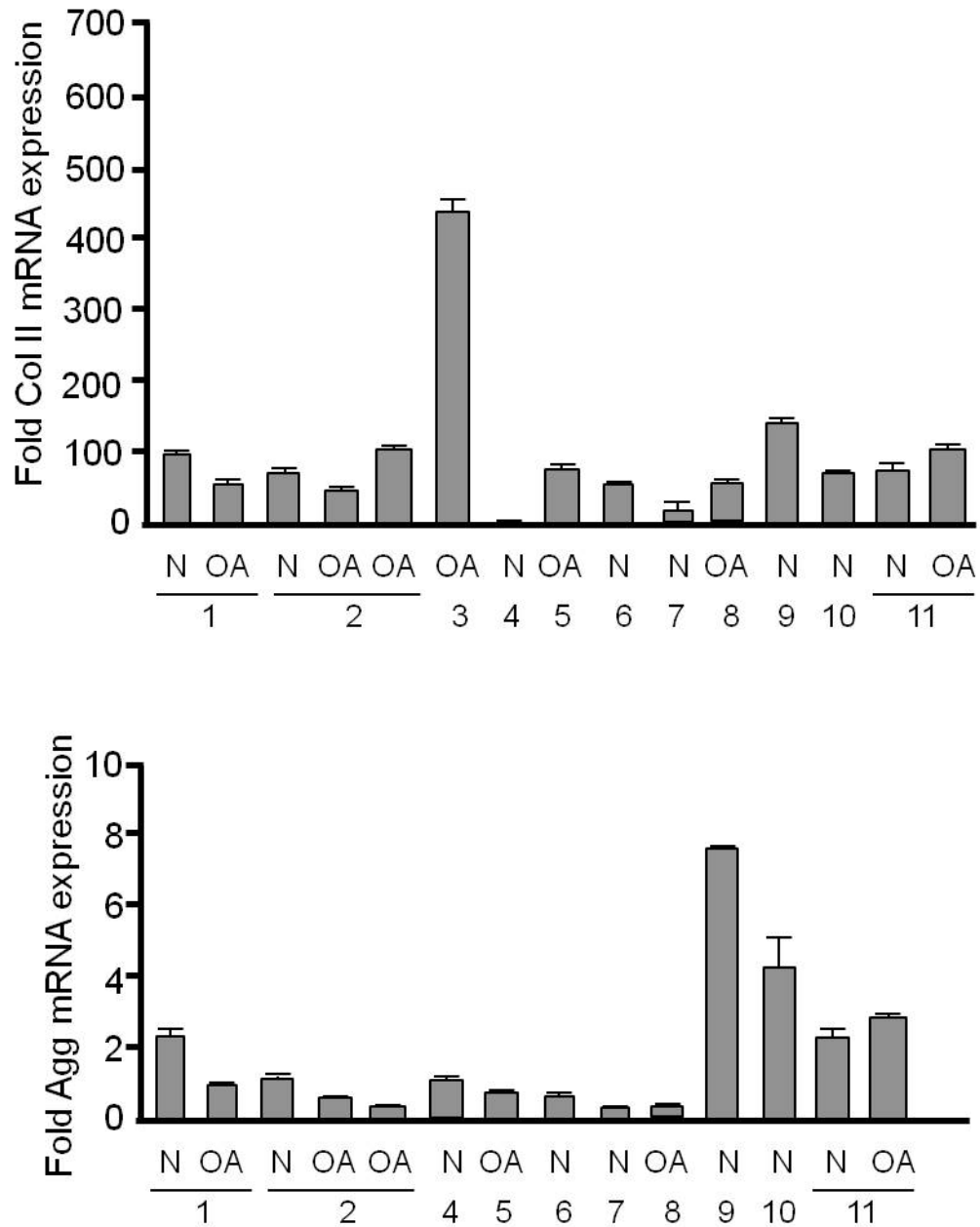
Gene	Sense primer	Annealing
size	Antisense primer	temperature
Collagen type II	5' ATGACAATCTGGCTCCCAAC (+4062)	62°C
(220 bp)	5' CTTCAGGGCAGTGTACGTGA (+4282)	
Collagen type X	5' TGAGCAGCAACGTAAAAACG (+2303)	62°C
(225 bp)	5' AGGAAATGCCGAGTTTCTCA (+2527)	
Aggrecan	5' TGAGTCCTCAAGCCTCCTGT (+6423)	62°C
(200 bp)	5' GGCATATGACGTGTCCCTCT (+6622)	
ALP	5' CCACGTCTTCACATTTGGTG (+1359)	62°C
(195 bp)	5' AGACTGCGCCTGGTAGTTGT (+1554)	
EF1 $\alpha$	5' AAAATGACCCACCAATGGAA (+1051)	62°C
(210 bp)	5' GCAGCATCACCAGACTTCAA (+1261)	

Table 6. Primers utilized in the PCR reactions following restriction enzyme digestion

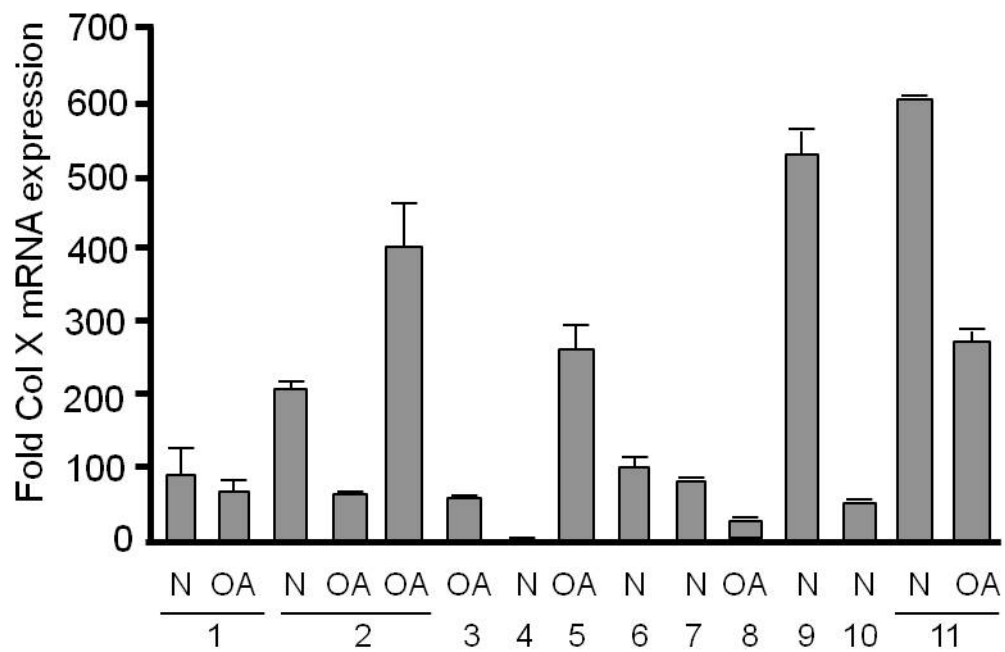
Gene	Sense primer	Annealing
size	Antisense primer	temperature
Collagen type II	5' CCTAGCATTTCCCCCTCTCC (+2932)	64°C
(549 bp)	5' TCCTCCAAGACTGCAGAGGAA (+3481)	
Collagen type X	5' TTGTTGCAATAGCCACAGATG (-1763)	64°C
(240 bp)	5' TGGATGCTTTTGAATGCTAGG (-1523)	



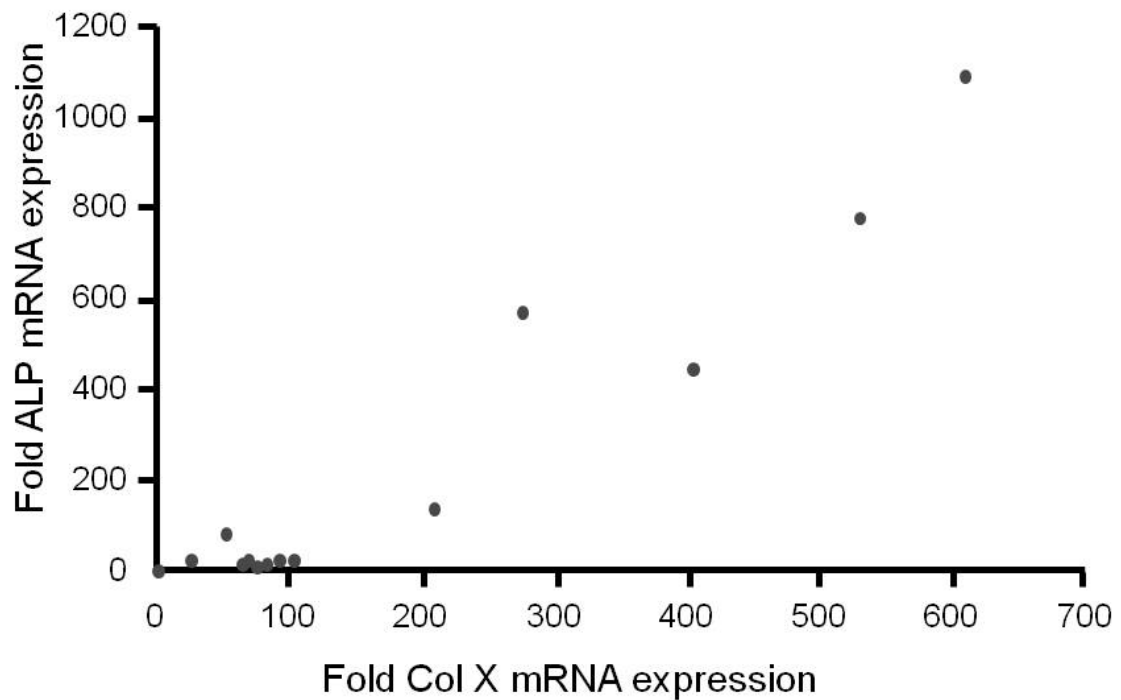
**Figure 13.** Restriction digestion analysis of Collagen type X promoter methylation. The methylation-sensitive restriction enzyme HpyCH4IV cuts unmethylated DNA templates but is unable to cleave methylated templates. As a consequence, PCR amplification of unmethylated DNA with primers that span the putative cleavage sites will be unsuccessful (at left) whereas methylated DNA will remain intact and support PCR amplification (at right).



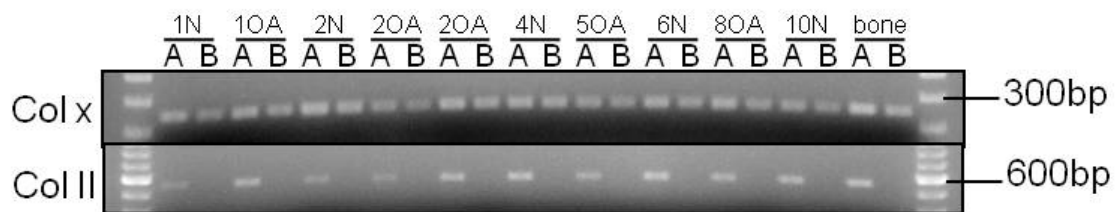
**Figure 14.** Collagen type II (Coll II; above) and aggrecan (Agg; below) mRNA expression in 15 articular cartilage specimens collected from 11 TJA patients. mRNA levels were measured by quantitative PCR, normalized to EF1a mRNA levels and presented as ‘fold increases’ above expression in the normal cartilage sample from patient #4.



**Figure 15.** Collagen type X (Col X) mRNA expression in 15 articular cartilage specimens collected from 11 TJA patients. Collagen type X mRNA levels were measured by quantitative PCR, normalized to EF1 $\alpha$  mRNA levels and presented as ‘fold increases’ above expression in the normal cartilage sample from patient #4. Note variation in collagen type X expression among individuals and within different areas from the same individual.

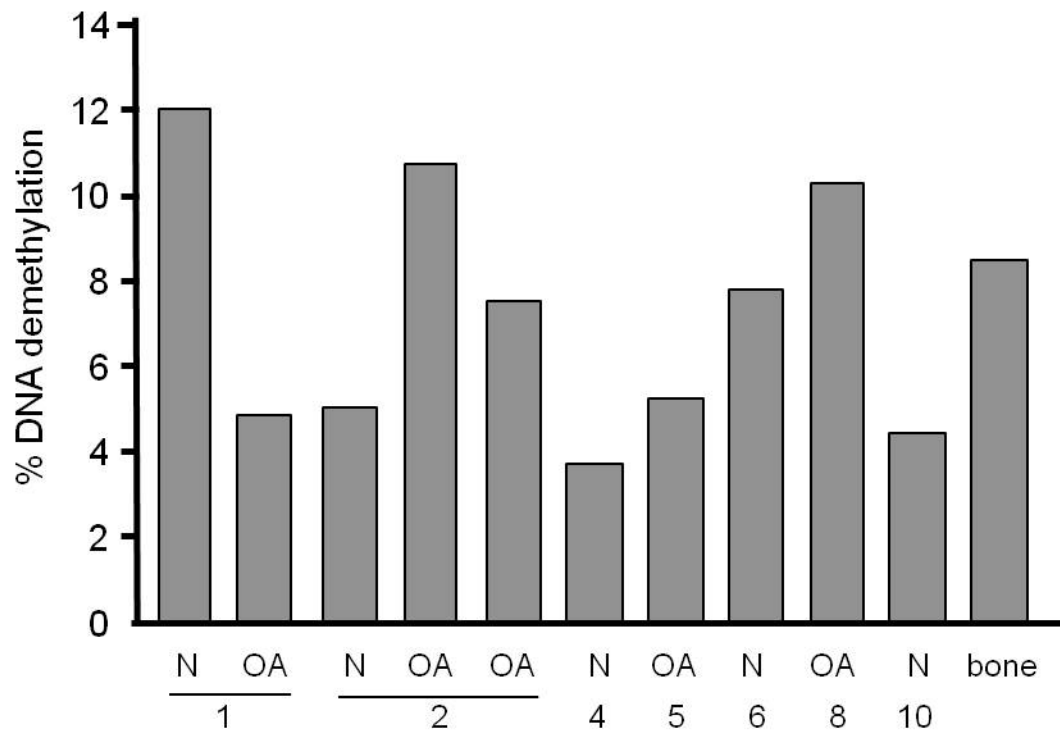


**Figure 16.** Relationship between collagen type X (Col X) mRNA expression and alkaline phosphatase (ALP) mRNA expression. Notice the broad expression ranges of both genes and the strong correlation between Col X and ALP expression ( $p = 0.0343$ ) in samples with Col X mRNA expression greater than 100 times the reference value (Pearson  $r = 0.5886$ ;  $R^2 = 0.3464$ ).

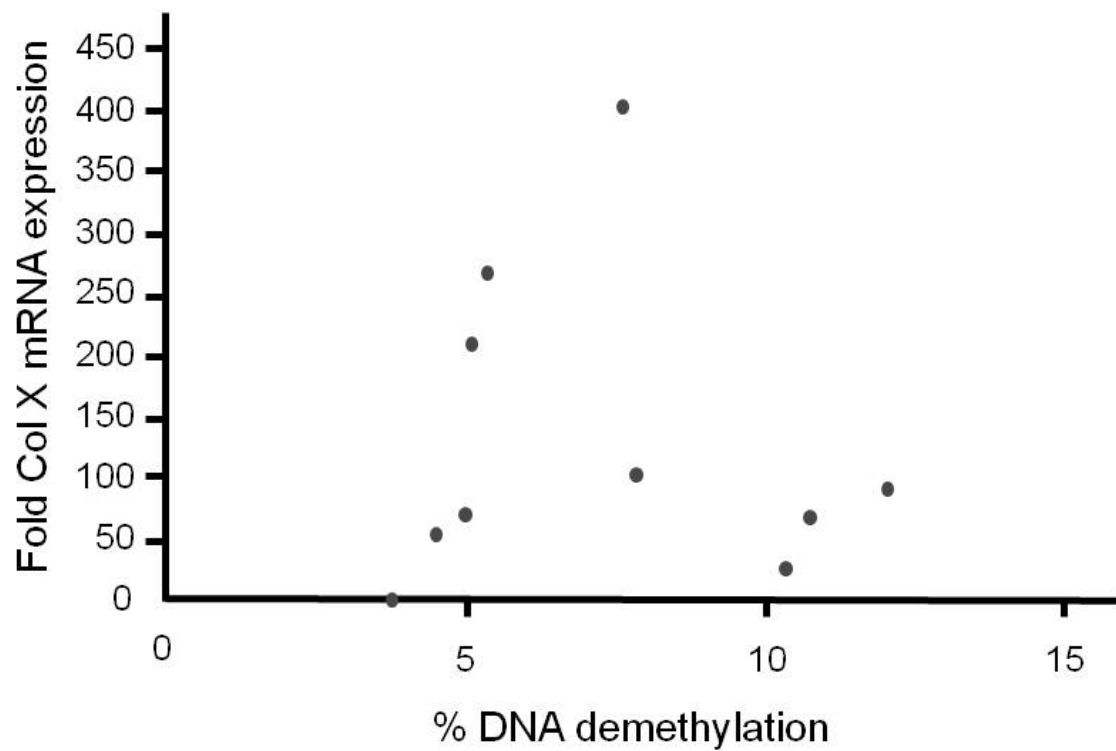


**Figure 17.** Gel image of Col X promoter amplicons generated from methylation-sensitive restriction enzyme digestion followed by PCR amplification. Lane A samples were digested with EcoRV. Lane B samples were digested with EcoRV and HpyCH4IV. Col II was used as a control for complete digestion. The differences in intensities between Col X 'A' and 'B' bands reflect variations in the amount of methylation among individuals and among different samples from the same individual.





**Figure 18.** Col X CpG demethylation based on quantification of band intensity after methylation-sensitive restriction enzyme digestion followed by PCR amplification approach. Notice that there is variation of amount of demethylation among individuals and among different samples from the same individual, but the level of demethylation, in general, was low among all the samples studied (below 12%).



**Figure 19.** The correlation between fold change in Col X mRNA expression and the level of demethylation in the same samples. The level of Col X mRNA expression is not correlated with the level of demethylation of the samples (Pearson  $r = -0.1452$ ;  $R^2 = 0.02107$ ).

## **CHAPTER 5: DIFFERENTIAL PHENOTYPIC EFFECTS OF 5-AZACYTIDINE ON ARTICULAR AND ENDOCHONDRAL CHONDROCYTE POPULATIONS**

### **INTRODUCTION**

Cartilage is a highly specialized avascular and aneural connective tissue characterized by a small number of cells, termed chondrocytes, interspersed within a large volume of extracellular matrix. Cartilage exists as a temporary tissue in cartilage anlagen during skeletal development, in the growth plates and epiphyses of growing bone and in fracture callus<sup>1-3</sup>. In these cases, the transient cartilage template is replaced by bone during the process of endochondral ossification. Collectively, signaling pathways mediated by the secreted ligands parathyroid hormone-related peptide (PTHrP), fibroblast growth factors (FGF), Indian hedgehog (Ihh), transforming growth factor beta (TGF- $\beta$ ), bone morphogenetic proteins (BMP), and Wnts interact to drive chondrocyte proliferation and to control the transition of chondrocytes into the hypertrophic phase of differentiation<sup>4-7</sup>. Hypertrophic chondrocytes undergo marked increases in cellular volume and length and express several phenotype-specific genes; in articular, collagen type X (Col X) and alkaline phosphatase (ALP). Terminally, hypertrophic chondrocytes undergo apoptosis and the hypertrophic cartilage matrix is colonized by vascular endothelial cells and osteoblast precursors<sup>4,7</sup>.

In contrast to the cartilaginous tissues engaged in bone formation, permanent cartilage is found in several parts of the body and is categorized into three types depending on differences in extracellular matrix composition. Elastic cartilage is rich in elastin and it is found in the ear pinna, epiglottis and larynx. Fibrocartilage contains

comparatively high levels of collagen type I and is located in intervertebral discs, meniscus, compressive sites within tendons and the pubic symphysis. Hyaline cartilage is rich in collagen type II (Col II) and proteoglycans and is located in the rib cage, nose, larynx, trachea, and bronchi, and on the articular surfaces of bones.

Articular cartilage is a highly specialized tissue that covers the articulating surfaces of bones. It is a hypocellular, avascular tissue, rich in extracellular matrix composed mainly of water, Col II and proteoglycans. The collagen fibrils provide the cartilage with tensile strength and resistance to surface shear forces, while the aggrecan: proteoglycan complexes provide resistance to compressive loads<sup>8,9</sup>. Articular chondrocytes are insensitive to hypertrophy-stimulatory signals active in endochondral chondrocyte populations and do not undergo hypertrophic differentiation under normal circumstances. Instead, articular chondrocytes persist throughout life in a nominally pre-hypertrophic state, characterized by the expression of Col II and aggrecan (Agg), with little or no Col X expression or ALP activity<sup>10</sup>.

The mechanism by which articular chondrocytes avoid hypertrophy is still not completely understood. There are several studies that indicate the transforming growth factor- $\beta$ <sup>11-13</sup>, bone morphogenetic proteins<sup>14-16</sup>, and parathyroid hormone related peptide<sup>17-20</sup> signaling pathways, mediated by lineage-specific transcriptional factors<sup>21-29</sup> are crucial to maintenance of articular cartilage and articular chondrocytic phenotype.

In recent years, there has been an increasing recognition that epigenetics play a role in cell differentiation and phenotype commitment. Epigenetics refers to “mechanisms that initiate and maintain patterns of gene expression and gene function in a heritable manner without changing the sequence of the genome”<sup>30</sup>. Epigenetics impact

gene regulation through DNA methylation, histone protein modifications and chromatin organization. DNA methyltransferases methylate cytidines at the CpG dinucleotide sequences. This modification can interfere with the recognition of binding sites by DNA binding proteins. Additionally, methyl-CpG binding proteins recruit histone deacetylases (HDACs), histone methyltransferases (HMTs) and chromatin-remodeling factors. These modifications condense chromatin structure, hiding potential binding sites for transcriptional activators and restricting occupancy. As a consequence, transcription is repressed. MicroRNAs are also involved in DNA methylation in *Arabidopsis thaliana*<sup>31</sup> and might also be involved in regulating the histone deacetylase HDAC4 in murine developing cartilage<sup>32</sup>. Recent findings suggest that at least 30% of genes are regulated by microRNAs; however, at this stage the epigenetic role of microRNA is predominantly indirect.

Epigenetic processes are involved in regulation of germline-specific genes, tissue-specific genes, imprinting, and aging<sup>30,33</sup>. In addition, experiments using inhibitors of the DNA methyltransferases have induced mouse embryo fibroblasts to differentiate into muscle, adipose tissue and chondrocytes<sup>34-36</sup>, and to inhibit differentiation of spermatogonia into spermatocytes<sup>37</sup>. These findings suggest that DNA methylation is involved in the regulation of gene expression during cell differentiation and lineage commitment<sup>38-40</sup>.

Epigenetic pathways also act in maintenance of the chondrocytic phenotype. The transcription factor Sox-9 is a master regulator of several chondro-specific genes<sup>21-29</sup>. Tsuda and co-workers demonstrated that the histone acetyltransferase (HAT), CBP/p300, associates with Sox-9 to regulate transcription of the Col II gene in chondrocytes<sup>41</sup>. p300

increases Sox-9 dependent transcription by increasing acetylation of histone amino tails in chromatinized DNA templates *in vitro*. In addition, trichostatin A, a histone deacetylase inhibitor, stimulates expression of Col II and aggrecan, and increased nucleosomal acetylation around the Sox-9 binding site in chondrocytes<sup>42</sup>. Taken together, these results support a model whereby the gene-specific factor Sox-9 interacts with non-specific chromatin-remodeling factors to activate Col II transcription in chondrocytes.

HDAC4 null mice exhibit premature chondrocyte hypertrophy and ossification, a phenotype similar to mice that overexpress RUNX-2 in chondrocytes. In addition, constitutive expression of HDAC4 mimics the phenotype seen in RUNX-2 knockout mice<sup>43</sup>. RUNX-2 is a transcription factor necessary for hypertrophic differentiation of chondrocytes. *In vitro* studies showed that HDAC4 interacts with RUNX-2, inhibits RUNX-2 DNA-binding and decreases histone acetylation of the RUNX-2 promoter<sup>43</sup>. Collectively, these data support a model in which HDAC4 negatively regulates chondrocyte differentiation by suppressing the activities of RUNX-2 with consequent inhibition of gene expression required for hypertrophy. In this model, HDAC4 is not acting in an epigenetic capacity, but is regulating the acetylation status of a non-genomic substrate. HDAC activity is also necessary to support collagen type II expression in articular chondrocytes, and permit re-differentiation of chondrocytes de-differentiated in monolayer<sup>44</sup>. In this study, collagen type II expression was linked to suppression of Wnt-5a expression by HDACs; again, a likely non-epigenetic activity.

DNA methylation also influences the chondrocytic phenotype. Phenotypically stable, chick articular chondrocytes, cultured as monolayers and treated with the DNA

methyltransferase inhibitor, 5-azacytidine (5-aza), adopted a hypertrophic phenotype, expressing Col X, Indian hedgehog (Ihh), and ALP <sup>45</sup>. Similarly, bovine fetal chondrocytes cultured as monolayers expressed hypertrophic markers in response to 5-aza treatment. Chondrocytes exposed to 5-aza for 48 hours decreased collagen type II expression and upregulated hypertrophic marker expression such as ALP, Col X, along with PTHrP and its receptor <sup>46</sup>. In addition, human articular chondrocytes cultured in alginate beads and exposed to 5-aza undergo phenotypic changes that resemble hypertrophy, including increases in cell size and apoptotic death <sup>47</sup>, consistent with transition from an articular to an endochondral phenotype.

Although the results of the 5-aza/chondrocyte studies mentioned above provide persuasive evidence for a role for CpG methylation in the regulation of articular and endochondral lineages, these studies utilized incompletely committed cell populations or, with the exception of Ho *et al*, culture models that intrinsically compromise the differentiated phenotype of chondrocytes. The objective of this study was to assess the phenotypic effects of 5-aza in healthy and fully committed articular and endochondral (growth plate) chondrocytes isolated from the same donors, under culture conditions that support the differentiated chondrocytic phenotype, to determine whether 5-aza can induce spontaneous hypertrophy or increase hypertrophy-responsiveness in these distinct chondrocyte populations. Two questions were addressed. Firstly; does 5-aza administration directly stimulate expression of hypertrophic genes in articular and growth plate chondrocytes? Secondly, does 5-aza administration alter the phenotypic responses of articular and growth plate chondrocytes to exogenous BMP-2 administration?

## **METHODS**

### **Sample collection and cell isolation**

Articular cartilage was collected from the femorotibial joints of three young adult horses (3-5 years of age) with no clinical evidence of arthritic disease. In addition, equine articular and growth plate cartilages were collected from three neonatal horses, within the first week of life, euthanatized for reasons other than musculoskeletal disease. Within this age range, the growth plate cartilage plates of the major long bones are easily separable from the adjacent epiphyseal and metaphyseal bone fronts. Articular cartilage was excised from the femorotibial, scapulohumeral and humeroradial joints. The epiphyses and adjacent metaphyses of the humerii, femora, proximal tibia and distal radii were then sectioned longitudinally at 5-mm intervals, using an oscillating bone saw, to create full-width slabs of the epiphyseal/growth plate/metaphyseal tissues. The growth plate cartilage was then separated from the adjacent bone by fracturing the chondro-osseous interface.

The cartilage specimens were diced, washed in phosphate buffered saline (PBS) (HyClone, Logan, UT) and then transferred to 500-mL Erlenmeyer flasks containing 10 mL/g of cartilage of Trypsin-EDTA (Gibco, BRL-Life Technologies, Grand Island, NY) supplemented with penicillin G sodium (200 units/mL) streptomycin (200 µg/mL) (Gibco), 2.5 µg/mL of amphotericin B (Cellgro, Mediatech, Inc, Herndon, VA). The flasks were placed in a C24 Shaking Incubator (New Brunswick Scientific, Edison, NJ) at 180 rpm at 37°C for an hour. The pre-digestion solution was aspirated and the explants were rinsed with PBS, then resuspended in 10 mL per gram of tissue in 0.15%



collagenase II (Worthington Biochemical Corporation, Lakewood, NJ) reconstituted in Opti-MEM supplemented with penicillin G sodium (200 units/mL) streptomycin (200 µg/mL), 2.5µg/mL of amphotericin B and 2% of fetal bovine serum (Gemini Bio products, West Sacramento, CA) was added to the flask for the overnight digestion in the C24 Shaking Incubator at 180 rpm at 37°C.

After the overnight digestion, the cell suspensions were filtered through a 40-micron filter (Nalgene Company, Fisher Scientific, Pittsburgh, PA) into a 50-mL Falcon tube (Fisher Scientific). The flask and filter were rinsed with PBS to recover any residual cells. After filtration, the cells were pelleted by centrifugation (Beckman Coulter, Inc., Fullerton, CA) for 10 minutes at 290 rcf. The supernatant was aspirated and the cell pellet was re-suspended in PBS and pelleted following the same protocol to assure removal of residual collagenase. The wash step was repeated several times for the growth plate chondrocytes due to the increased amount of debris and blood. Finally, the cells were resuspended in 10-20 mL of Opti-MEM for cell counting and viability assessment.

### **Cell count and viability**

The cell numbers were calculated using a hemacytometer (Fisher Scientific) and inverted light microscope (Fryer Co. Inc., Huntley, IL). Two vials containing a 1:10 dilution of the cell suspension and 10% Trypan blue dye (Gibco) were prepared. The samples were well mixed and 10 µL were added to each side of the hemacytometer. Dead and live cells were counted in a total of 9 (1 mm<sup>2</sup>) squares in each side. The total number of cells in the cell suspension was obtained by the following formula:

# cells = # of cells counted/18 (# of 1 mm<sup>2</sup> squares counted) x 10 (cell dilution) x 10 or 20 (total volume of the cell suspension) x 10<sup>4</sup>

### **Culture conditions**

Three to four million chondrocytes were cultured as non-adherent aggregates in defined serum-free culture medium (Opti-MEM, Gibco) in six-well (10 cm<sup>2</sup>) hydrogel-coated, ultra low attachment culture plates (Corning Costar, Pittsburgh, PA). The medium was supplemented with ascorbic acid (Sigma-Aldrich, St. Louis, MO) (50 µg/mL) and penicillin G sodium (100 units/mL) streptomycin (100 µg/mL). Cultures were maintained at 37°C, in 95% air/ 5% CO<sub>2</sub> in a humidified incubator (Fisher Scientific). After 24 hours, the chondrocytes were treated with 10 µM of 5-azacytidine (Sigma-Aldrich) or maintained in basal medium (controls) for 4 days. On day 4, the 5-aza cultures were divided into two groups. The first group was treated continuously with 5-aza for an additional six to eight days (aza cont). The second group was washed with medium to remove the 5-aza and allow recovery, and then maintained in control media for the remaining culture period (aza rec). The control cultures (Cx), aza cont and aza rec cultures were additionally maintained in the presence or absence of 100 ng BMP-2/mL, to stimulate hypertrophy. In some experiments, 100 ng thyroid hormone (T3)/ml was also administered to stimulate hypertrophic differentiation. Chondrocyte aggregates were collected after 4 days of 5-aza exposure, and at the conclusion of the experiments, and were snap frozen in liquid nitrogen and stored at -80°C for further processing.

## **RNA isolation**

Total RNA was isolated from the chondrocyte aggregates using the phenol-based dissociation agent, TRIzol<sup>®</sup> (Invitrogen Corporation, Carlsbad, CA). One milliliter of TRIzol<sup>®</sup> was added to each frozen aggregate pellet and the sample was immediately homogenized using an Ultra-Turrax T25 homogeneizer (Janke & Kunkel, IKA-Labortechnik, Staufen, Germany) for 30 seconds. The samples were then centrifuged in a refrigerated centrifuge 5810 R (Eppendorf) at 453 rcf for 10 minutes to precipitate the insoluble debris. The TRIzol<sup>®</sup> supernatants were transferred to a 1.5-mL microcentrifuge tube (Eppendorf) and 200  $\mu$ L of chloroform per milliliter of TRIzol<sup>®</sup> was added to separate the aqueous and organic phases of the lysate. After vortexing, the tubes were centrifuged in a refrigerated centrifuge (Centrifuge 5415 R, Eppendorf) at 16,100 rcf for 30 minutes at 4°C. The upper aqueous phase (approximately 400  $\mu$ L) was removed and placed in a new 1.5-mL microcentrifuge tube. During this step, great care was taken to leave the insoluble material at the interface intact. This interface material is comprised of insoluble proteins, lipids, DNA and, with chondrocyte cultures, large amounts of proteoglycans. To maximize RNA yields, the remaining aqueous volume and a small amount of the adjacent interface was then transferred to a 0.5-mL microcentrifuge tube (Fisher). These smaller tubes were then centrifuged at 16,100 rcf and an additional 100-200  $\mu$ L of aqueous phase was transferred to the 1.5-mL microcentrifuge tubes.

The RNA was precipitated from the aqueous solution by addition of 250  $\mu$ L of isopropanol and 250  $\mu$ L of 1.2 M sodium chloride/0.8M sodium citrate. This high-salt precipitation protocol minimizes co-precipitation of sulfated proteoglycans. The microcentrifuge tubes were stored overnight at -20°C to facilitate RNA precipitation.

The samples were then centrifuged at 16,100 for 30 minutes at 4°C to pellet the RNA. The pellet was washed with 500 µL of 70% ethanol to reduce the salt content of the pellet, and allowed to air dry for 10 minutes. The pellet was dissolved in diethyl pyrocarbonate (DEPC)-treated water. A 1:40 aliquot of each sample was used to quantify the nucleic acid concentration spectrophotometrically (SmartSpec™ 3000, Bio-Rad, Hercules, CA), using optic density (OD) values at 260 and 320 nM. The concentration of total RNA was calculated as follow:

$$\text{RNA in } \mu\text{g}/\mu\text{L} = \frac{\text{OD at 260 nM} - \text{OD at 320 nM} \times 40 \text{ (dilution factor)} \times 40 \text{ (coefficient)}}{1000}$$

### **Northern blot analyses**

Four micrograms of total RNA were precipitated and loaded in a 1% agarose/formaldehyde gel to allow transcript size separation by electrophoresis in MOPS buffer as previously described<sup>48</sup>. The electrophoresed RNA was transferred from the gel onto nylon membranes (GE Infrastructure, Minnetonka, MN) by overnight capillary transfer using a high-salt (10X SSC) transfer solution. The transferred RNAs were immobilized to the nylon substrate by UV irradiation crosslinking (Spectrolinker XL-1000 UV crosslinker, Spectronic Corporation, Westbury, NY).

For Northern blot hybridization, membranes were re-hydrated, rinsed in 5xSSPE and then pre-hybridized at 65°C in a rotating hybridization oven for 1-2 hours in pre-hybridization solution (5X SSPE, 5 X Denhardt's solution (Eppendorf), 0.25% SDS (Fisher Scientific), 10% dextran sulfate (Fisher Scientific)) and 150 µg/mL denatured salmon sperm DNA (Invitrogen). Isotopic probes were prepared by random-primed labeling, using a commercial labeling kit (Prime-it® II, Stratagene, Cedar Creek, TX) and

<sup>32</sup>P-dCTP (Amersham, Piscataway, NJ). The probes were purified using G-50 Sephadex spin columns (Boehringer-Mannheim, Indianapolis, IN), denatured and then added to hybridization tubes containing the pre-hybridized membranes.

After overnight hybridization, the membranes were rinsed in 2X SSPE, 0.5% SDS. Sequential washes were done until background activity was minimal, as assessed by a handheld Geiger counter GSM-110 (Wm. B. Johnson & Associates, Inc., Fairlea, WV). The membranes were wrapped in Saran plastic (Jonhson & Son Inc., Racine, WI) and exposed overnight to a phosphor screen (Packard Instrument Comp, Inc., Meridan, CT) for quantitative analyses of signal intensity with Cyclone Storage Phosphoscreen (Packard). Finally, the membranes were exposed to autoradiograph film (Kodak Bio-Max MS Film, Carestream Health, Inc., Rochester, NY) for “hardcopy” recording of membrane activity. Radiograph cassettes were stored overnight at –80°C. Dr Dean Richardson generously provided the equine collagen type X cDNA used as a probe template for these experiments. The EF1 $\alpha$  cDNA used for Northern blot probe synthesis was generated by the primers listed in Table 7.

### **Reverse transcription**

First strand cDNA was synthesized by reverse transcription using First Strand cDNA Superscript II (Invitrogen) and Oligo dT as per manufacturer’s instructions. Briefly, one microgram of total RNA was brought up to 10  $\mu$ L and then combined with 1  $\mu$ L of oligo (dT) (0.5 $\mu$ g/ $\mu$ L) and 1  $\mu$ L of 10 mM dNTPs. The sample was incubated in a 65°C water bath for 5 minutes and then placed on ice for 1 minute to denature the RNA and allow binding of the oligonucleotides. The following reagents were added: 2  $\mu$ L 10x

First Strand Buffer, 2  $\mu$ L 0.1M DTT, 2  $\mu$ L 50 mM  $MgCl_2$  and 1  $\mu$ L RNase out. After a gentle mix and brief centrifugation, the sample was incubated at 42°C water bath for 2 minutes, 1  $\mu$ L (50 units) of SuperScript II Reverse Transcriptase was added. The sample was then incubated at 42°C for 50 minutes. The reaction was stopped by placing the sample in a 70°C water bath for 15 minutes. The RT reactions were diluted (1:10 ratio) with water and stored at -20°C until further use.

### **Quantitative polymerase chain reaction (qPCR)**

Quantitative PCR (qPCR) was used to compare Col X and ALP gene expression profiles of articular and growth plate chondrocytes samples. The PCR reactions were carried out in a final volume of 25  $\mu$ L. The 2x SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) was used for these reactions. This reagent contains 100 nM KCl, 40 mM Tris-HCl, 0.4 mM of each dNTP, 50 units/mL of *Taq* polymerase, 6 mM  $MgCl_2$ , and 20 nM SYBR Green I. The SYBR Green Supermix (12.5  $\mu$ L) was added to 1  $\mu$ L of the sense (10 pMol) and antisense primers. Autoclaved distilled water (5.5  $\mu$ L) and 5  $\mu$ L of cDNA template were also added. PCR conditions were: initial denaturation step at 95°C for 3 minutes, followed by 45 cycles of denaturation at 95°C for 10s, annealing temperature of 62°C for 30s and polymerase extension at 72°C for 20s. The samples were denatured at 95°C for 1 min before starting the melting curve protocol that consisted of decreasing the temperature at 55°C for 1 minute followed by increments of 0.5°C every 10 s till 95°C was reached. The primers are listed in Table 7. The primer combinations used in these experiments were designed for annealing at 60-62°C, however optimal annealing temperatures were determined by temperature gradient

analyses spanning 54-64°C. The specificity of the PCR products was monitored by melting curve analyses, PCR fragment cloning and sequencing. Graphs showing the fluorescence intensity were plotted for each cycle. Quantitative results were accessed using the cycle threshold determined to represent the onset of the linear phase of amplification, as calculated by the thermal iCycler software (Bio-Rad). To exclude contamination, water was used as negative control for each on the reactions instead of cDNA. PCR amplification of each sample was run in duplicate and qPCR data was normalized by the elongation factor-1 alpha (EF1 $\alpha$ ) expression.

### **Statistical analyses**

Differences between Col X and ALP mRNA levels after 12 days in culture (and after 8 days of control or BMP-2 exposure) were assessed by one-way ANOVA. Were significant differences were identified ( $p < 0.05$ ), Bonferoni's post hoc tests were used to identify specific effects of 5-aza treatment and BMP-2 administration.

## **RESULTS**

### **Phenotypic effects of 5-aza on adult articular chondrocytes**

Treatment of fully differentiated articular chondrocytes from adult horses with 5-azacytidine did not stimulate phenotypic change suggestive of hypertrophic differentiation. As seen in Figure 20A, initial treatment with 5-aza for four days did not induce collagen type X expression and Col X transcripts were undetectable in both control and aza-treated samples after 6 days treatment with BMP-2 or thyroid hormone. Both the agents are potent stimulators of hypertrophy in competent chondrocyte

populations. In a similar experiment that assayed persistent and transient (4 day) 5-aza treatment in conjunction with BMP-2 administration, neither Col X nor ALP expression was significantly affected (Figure 20B). The threshold cycles for these qPCR outcomes were consistently above 30 cycles; approximately 10 cycles above threshold for Col X and ALP expression in endochondral-competent chondrocyte populations.

### **Phenotypic effects of 5-aza on neonatal chondrocytes**

Articular and growth plate chondrocytes were collected from three equine neonates, within the first few days of life. In fact, the third foal in this study was obtained through a dystocia-related death of the mare. As a consequence, this foal had not been ambulatory prior to the tissue collections. To ensure that the articular chondrocytes collected from these foals were phenotypically consistent, the behavior of these cell populations under control culture conditions was assessed. The results of these analyses using cells from the third donor foal are shown in Figure 21. The data from both articular and growth plate chondrocytes were normalized to EF1 $\alpha$  mRNA levels to allow direct comparison. As is clearly evident, neither chondrocyte type underwent spontaneous differentiation in basal medium. BMP-2 administration induced substantial collagen type X and ALP up-regulation in growth plate chondrocytes after 8 days, whereas articular chondrocytes expressed no evidence of hypertrophic differentiation. On the basis of these findings, the effects of 5-aza on articular and growth plate chondrocytes were assessed after 8 days of BMP-2 administration.

In control cultures, articular chondrocyte Col X expression was initially detectable at relatively low levels (threshold above 24 cycles) and expression fell during



time in culture. Initial exposure to 5-aza for four days did not significantly alter Col X levels. Neither removal of 5-aza from the culture medium after four days, nor persistent 5-aza for the subsequent 8 days of the experiments affected Col X expression. Finally, co-treatment with BMP-2 did not up-regulate Col X expression. Representative data from chondrocytes isolated from the second equine donor are presented in Figure 22 (upper panel).

Initial ALP expression in articular chondrocytes was consistently low (threshold over 32 cycles) and basal ALP expression did not change with time in culture. 5-aza treatment, whether transient or persistent, did not increase expression of ALP expression, in comparison to control levels. Representative data from chondrocytes isolated from the second equine donor are presented in Figure 22 (lower panel). BMP-2 administration did increase ALP expression of control cultures, albeit with considerable variability between the three donors (597-, 2.25- and 38.7-fold increases over control values). The qPCR cycle threshold cycles in these analyses were consistently at or above 30; arguably below levels of biological relevance. In contrast, both collagen type II and aggrecan were highly expressed in all samples (data not shown).

In growth plate chondrocyte control cultures, Col X expression fell over time. 5-aza treatment, whether transient or persistent, did not induce expression of Col X. As expected, Col X was up-regulated by BMP-2 treatment. Persistent 5-aza administration inhibited growth plate chondrocyte responses to BMP-2. In contrast, transient exposure significantly increased Col X induction by BMP-2. Representative data from chondrocytes isolated from the second donor foal is shown in Figure 23 (upper panel). Transient 5-aza administration increased Col X expression in response to BMP-2 in all

three experiments; however, this effect was variable, ranging from 1.5- to 16-fold increases.

Low levels of ALP expression were maintained throughout the experiments. 5-aza treatment, by itself, did not induce expression of ALP. Persistent 5-aza administration inhibited any response to BMP-2, as with Col X expression (above). Transient 5-aza expression resulted in modest but not significant increases in ALP expression in response to BMP-2 administration. Representative data from chondrocytes isolated from the second donor foal is shown in Figure 23 (lower panel). As with the articular cartilage samples, collagen type II and aggrecan were consistently expressed in all experiments (data not shown).

The extent to which 5-aza administration induced a phenotypic transition towards a hypertrophic/endochondral chondrocyte type is graphically represented in Figure 24, where expression of the phenotype-specific genes in both cell types has been normalized to EF1 $\alpha$ . In comparison to the Col X and ALP expression levels induced by BMP-2 in growth plate chondrocytes, expression of these hypertrophic markers by articular chondrocytes was consistently negligible. The results of this study do not indicate that 5-aza is able to induce hypertrophic transformation in articular chondrocytes, although 5-aza did variably increase the responsiveness to growth plate chondrocytes to BMP-2 administration.

## **DISCUSSION**

Epigenetics refers to “mechanisms that initiate and maintain patterns of gene expression and gene function in a heritable manner without changing the sequence of the

genome”<sup>30</sup>. Epigenetics impact gene regulation through DNA methylation, histone protein modifications and chromatin organization. Several studies have demonstrated the importance of epigenetics in stem cell differentiation and lineage commitment. For example, several germline-specific genes are methylated in fibroblasts but not in sperm<sup>33</sup>, suggesting that DNA methylation is involved in repression of germ-line specific genes in somatic cells, and participates in the epigenetic regulation of gene expression during cell differentiation<sup>38-40</sup>. Moreover, genome-wide screens have provided information on differential methylation in different types of tissues<sup>39, 49-52</sup>.

The DNA methyltransferase inhibitors are derivatives of 5-azacytidine (5-aza). 5-aza is a cytidine analog modified at position 5 that cannot be methylated. It was originally synthesized by Piskala and Sorm in 1964 with the objective to treat acute myelogenous leukemia<sup>53</sup>. More recently, 5-aza and several other cytidine analogues, such as 5-aza-2-deoxycytidine (5-aza-CdR), zebularine, and decitabine, among others, have been used to treat several neoplastic diseases<sup>54, 55</sup>. DNA methyltransferase inhibitors have been used to experimentally address the effects of DNA methylation on cellular differentiation and phenotypic stability. For instance, DNA methyltransferase inhibitors induce mouse embryonic fibroblasts to differentiate into muscle, adipose tissue and chondrocytes<sup>34-36</sup>. In addition, 5-aza-deoxycytidine (5-aza-CdR), prevents differentiation of spermatogonia into spermatocytes in mouse<sup>37</sup>, which suggests that DNA methylation is important for terminal differentiation of spermatocytes. On the other hand, the treatment of articular chondrocytes with DNA methyltransferase inhibitor alleviates molecular constraints that prevent these cells from achieving terminal maturation<sup>45</sup>. It should be noted that, in many

of these differentiation-focused studies, the specific mode of 5-aza action was not identified and alterations in DNA methylation were not directly addressed.

In this study, we investigated whether 5-aza could induce expression of hypertrophic genes in equine articular and growth plate chondrocytes, and whether 5-aza administration could affect the phenotypic responses of equine articular and growth plate chondrocytes to exogenous BMP-2 administration.

All cells presented high levels of expression of the chondrocyte-specific genes aggrecan and collagen type II. This suggests that the chondrocytic phenotype was maintained throughout the experiments. This is not surprising since the culture system used is known to maintain the chondrocytic phenotype<sup>16, 56</sup>. Articular chondrocytes expressed very little Col X or ALP mRNAs. Further, articular chondrocytes did not upregulate either of these hypertrophic genes in response to BMP-2. Moreover, 5-aza treatment did not induce expression of the hypertrophic genes or altered the articular chondrocytes' response to the hypertrophic effects of BMP-2. It is possible that the disparity between our results and Zuscik *et al*'s findings could be explained by the difference in cell culture conditions. Zuscik *et al* cultured chondrocytes as monolayers and in the presence of serum. These culture conditions induce phenotypic dedifferentiation, markedly altering cell shape and down-regulating expression of chondrocyte-specific genes such as collagen type II and aggrecan<sup>16, 56</sup>. It is also known that serum contains several growth factors and also interfere with the phenotypic stability of chondrocytes. These model-linked factors could have increased the cells' ability to respond to 5-aza and undergo hypertrophy. The study by Zuscik *et al* used chondrocytes isolated from the articular and epiphyseal regions of chick tibiae. Given that skeletal

development and growth in avian species differs considerably from mammalian growth in several respects, the difference in 5-aza responses may reflect differing phenotypic characteristics of immature avian and mammalian ‘articular’ cell populations; Zuscik *et al*’s avian articular cells behaved very similarly to the equine growth plate cells. It is also possible that the non-adherent aggregate culture model used in our study supported insufficient proliferation to permit 5-aza genomic incorporation, although previous studies have established that chondrocytes maintained in aggregate cultures undergo at least one population doubling during the first 3-4 days in culture. Generally, only 5% of cytidines are substituted by 5-aza, but this degree of substitution is sufficient to induce a global decrease in genomic DNA methylation (80-85%), due to consequent covalent binding of Dnmt1<sup>57, 58</sup> which effectively sequesters the enzyme and leads to a global reduction in genomic methylation<sup>59, 60</sup>.

As reported by Ho *et al* 2006, human articular chondrocytes cultured in alginate beads undergo phenotypic changes that resemble hypertrophy, following 5-aza treatment, with up-regulation of Col X and ALP expression and down-regulation of collagen type II<sup>47</sup>. These results also contradict the outcomes of the current study; however the up-regulation of Col X and ALP after 20 days in culture in Ho *et al*’s study, two-fold and 10-fold respectively, were modest when expression levels of bona fide endochondral chondrocytes are considered and are substantially less than the alteration in hypertrophic marker expression seen in osteoarthritic articular chondrocytes (see Chapter 4). From a quantitative perspective, it is difficult to accept that conclusions of Ho *et al* that 5-aza administration resulted in hypertrophic differentiation of human articular chondrocytes.

Equine growth plate chondrocytes were used to determine whether 5-aza augments the hypertrophic response of endochondral chondrocytes. In control cultures Col X expression decreased over time while ALP expression was maintained at low expression levels throughout the experiment. As expected, both Col X and ALP were up-regulated by BMP-2 treatment. Similarly to articular chondrocytes, 5-aza treatment, by itself, did not increase expression of the hypertrophic genes Col X or ALP. However, pre-treatment with 5-aza increased Col X up-regulation in response to BMP-2, compared to control samples. These results are consistent with the findings of Cheung *et al* 2001. In Cheung *et al*'s study, fetal bovine epiphyseal (endochondral) chondrocytes treated with 5-aza up-regulated hypertrophic gene markers and increased cell volume<sup>46</sup>. In a subsequent study, it was also shown that 5-aza induced chondrocyte apoptosis, another feature of terminal chondrocyte differentiation<sup>61</sup>. Collectively, these results suggest that 5-aza administration does increase the capacity of endochondral chondrocyte populations to undergo hypertrophic differentiation with appropriate stimulation.

Accepting this conclusion, no data are available to support a direct link between changes in genomic methylation status and consequent phenotypic alterations. 5-aza also influences cellular activity through induction of the DNA damage response, which leads to p53 activation, p21Waf1/Cip1 up-regulation and cell cycle arrest<sup>62</sup>. This mechanism could explain the increased chondrocyte apoptosis observed in Ho's study, for instance. Given that growth arrest is an integral feature of hypertrophic differentiation, the observed phenotypic effects of 5-aza on endochondral chondrocytes could relate to cell cycle regulation, as opposed to any epigenetic mechanisms.

In conclusion, the outcome of this study does not support the contention that interference with DNA methylation “trans-differentiates” articular chondrocyte populations into the endochondral lineage. In fact, fully differentiated articular chondrocytes were remarkably resistant to phenotypic effects of 5-aza. In contrast, transient 5-aza administration did augment the hypertrophic response of growth plate chondrocytes to BMP-2, suggesting that changes in genomic methylation profiles influence the progression of endochondral chondrocytes through hypertrophic differentiation.

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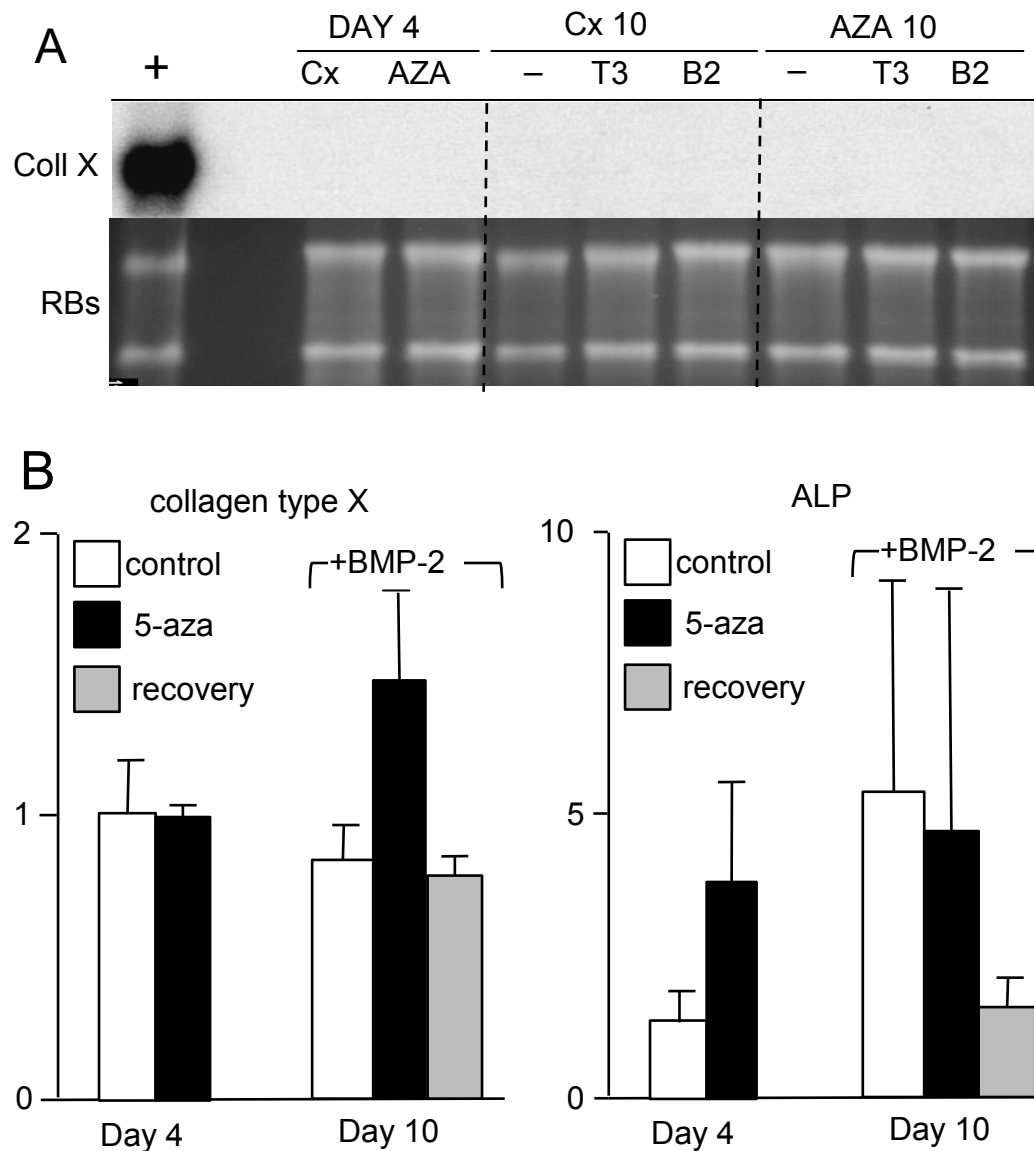
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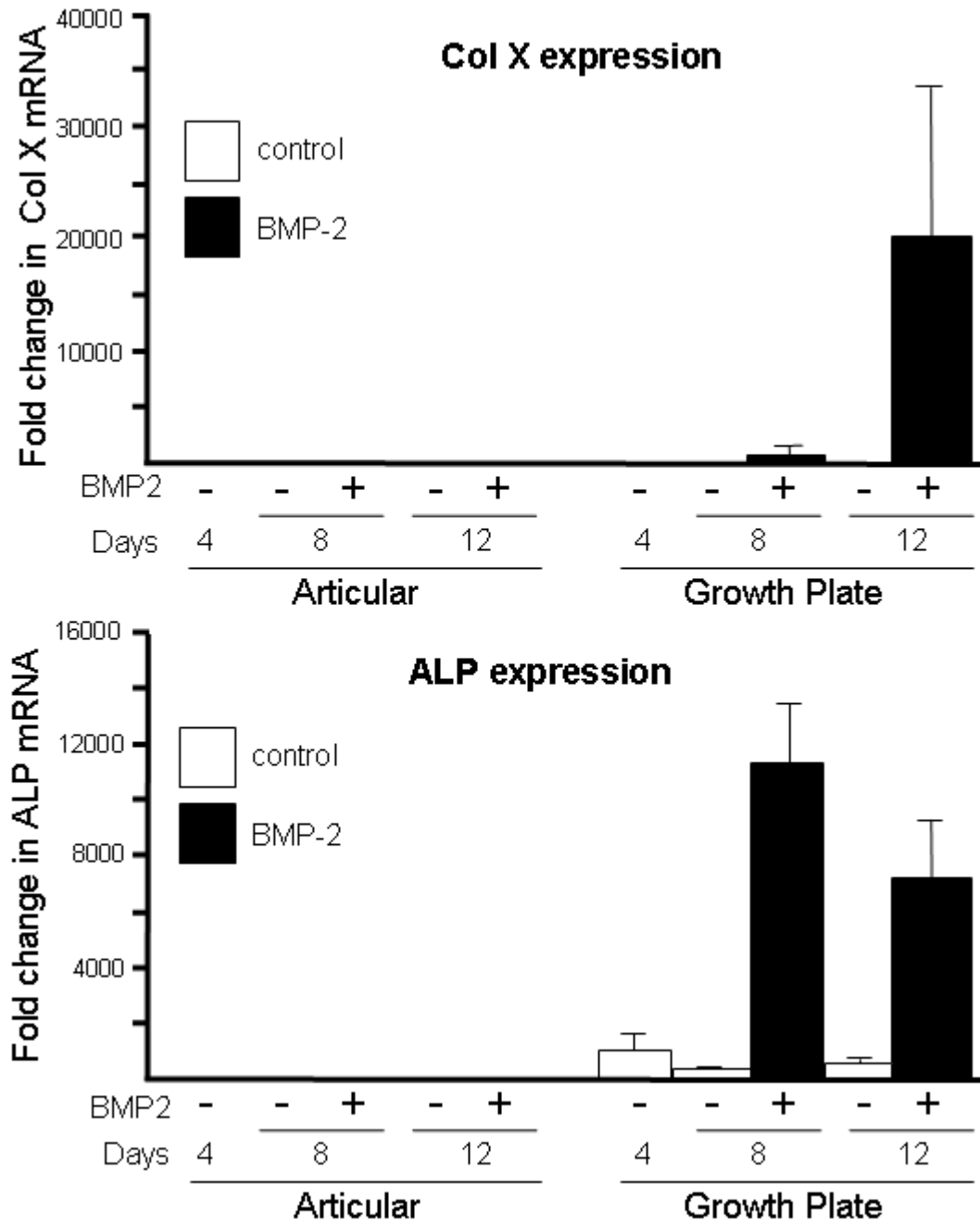
## FIGURES AND TABLES

Table 7. Primers utilized in the qPCR reactions

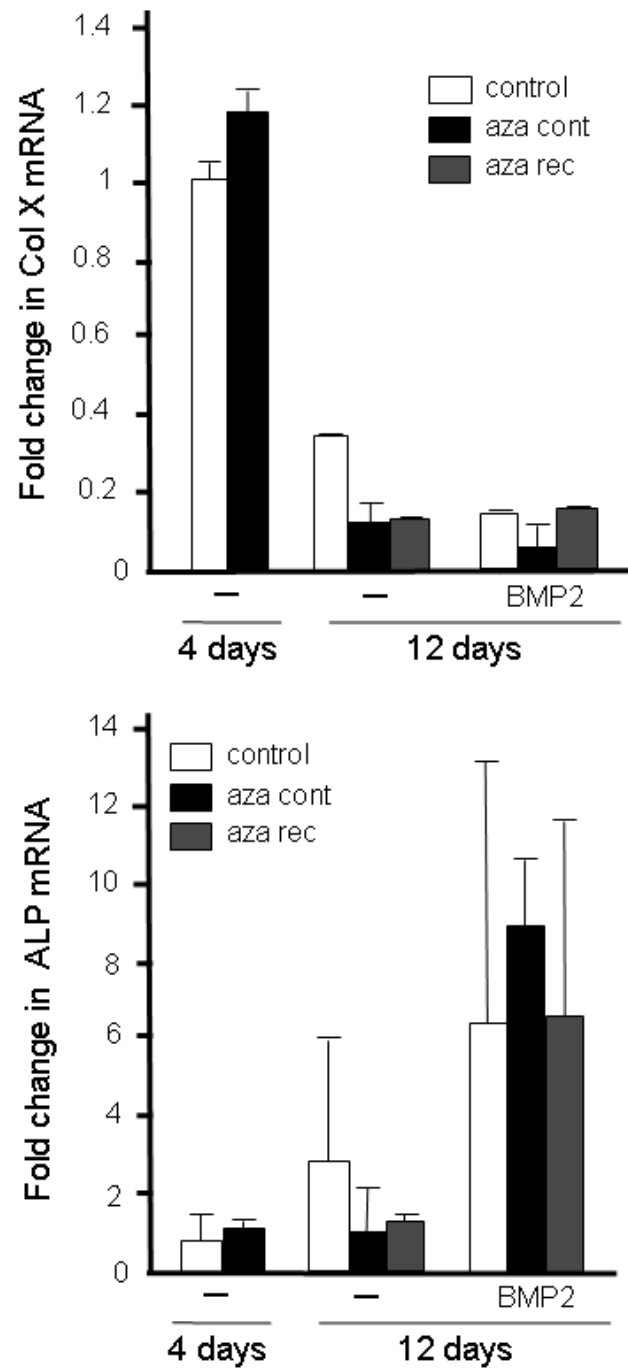
Gene	Sense primer	Annealing
size	Antisense primer	temperature
Collagen type II	5'AGCAGGAATTTGGTGTGGAC (4297)	62°C
(223 bp)	5'TCTGCCCAGTTCAGGTCTCT (4520)	
Collagen type X	5'TGCCAACCAGGGTGTAACAG (1605)	62°C
(244 bp)	5'ACATTACTGGGGTGCCGTTC (1840)	
ALP	5'TGGGGTGAAGGCTAATGAGG (357)	62°C
(221 bp)	5'GGCATCTCGTTGTCCGAGTA (578)	
EF1 $\alpha$	5'CCCGGACACAGAGACTTCAT (368)	62°C
(328 bp)	5'AGCATGTTGTCACCATTCCA (696)	



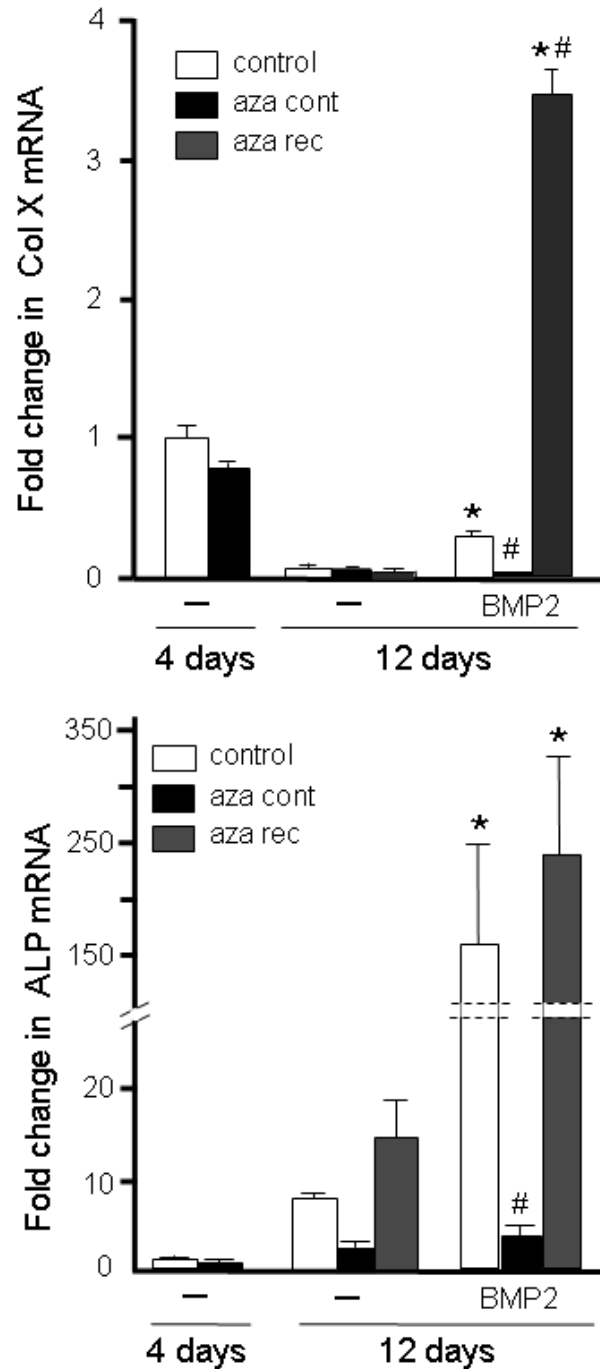
**Figure 20.** Response of adult articular chondrocytes to 5-azacytidine. **A.** Northern blot of Col X mRNA expression following 5-aza administration and subsequent treatment with thyroid hormone (T3) or BMP-2. Growth plate RNA was used as the positive control (+). **B.** qPCR analyses of Col X and ALP mRNA expression in response to BMP-2 under 'control' conditions, in the presence of '5-aza', and in 'recovery' cultures, where 5-aza was removed after 4 days. Y axes indicate fold changes in expression. There were no significant responses to BMP-2 following persistent or transient 5-aza administration.



**Figure 21.** Phenotypic distinctions between articular and growth plate chondrocytes isolated from equine neonates. qPCR analyses of Col X (upper panel) and ALP (lower panel) mRNA expression in the presence or absence of 100 ng BMP-2/ml. Responses by articular chondrocytes were negligible, whereas growth plate chondrocytes robustly increased both Col X and ALP expression in response to BMP-2 administration.

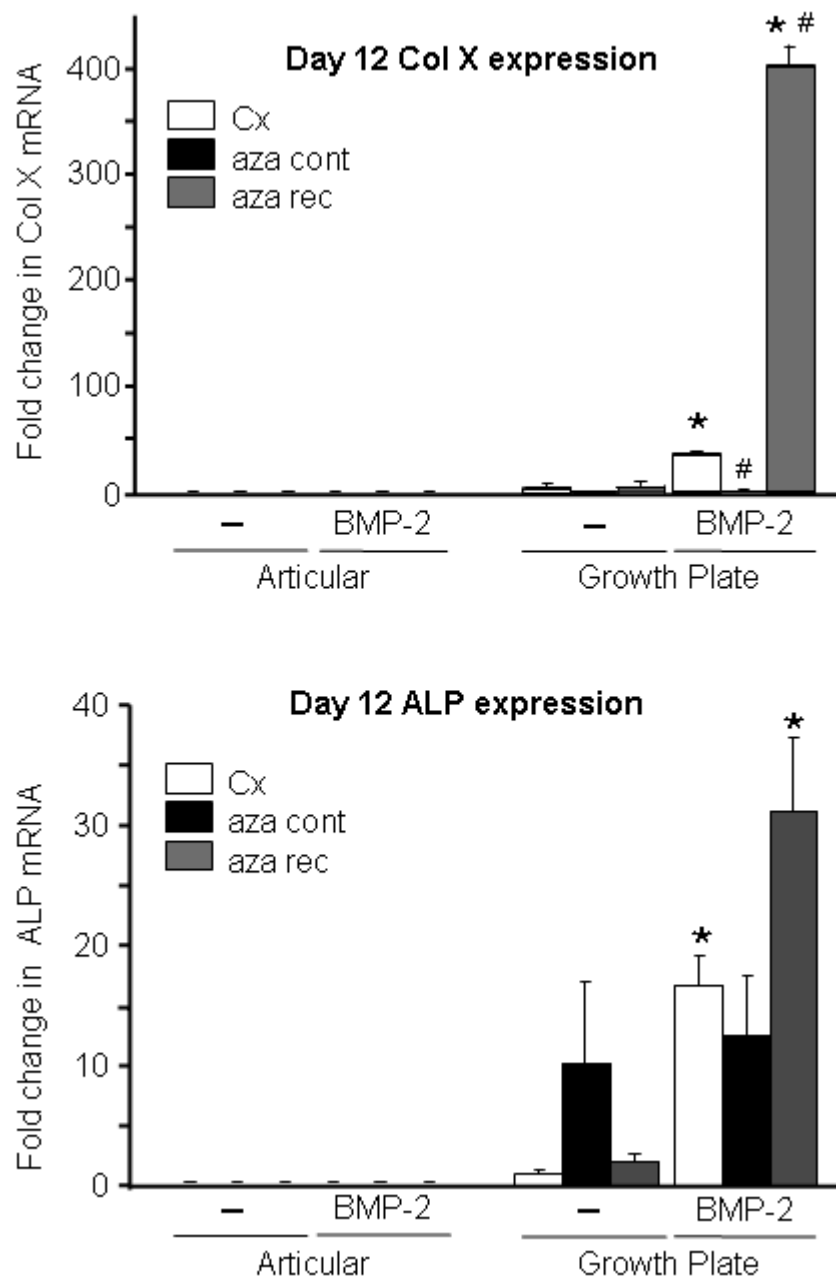


**Figure 22.** Response of neonatal articular chondrocytes, subject to continuous (aza cont) or transient (aza rec) 5-aza treatment, to BMP-2 administration. Expression of Col X and ALP mRNAs was assessed by qPCR.



**Figure 23.** Response of neonatal growth plate chondrocytes, subject to continuous (aza cont) or transient (aza rec) 5-aza treatment, to BMP-2 administration. Expression of Col X and ALP mRNAs was assessed by qPCR. Asterisks indicate a significant response to BMP-2 over the appropriate control sample. # indicate a significant effect of 5-aza over the appropriate control sample (ANOVA;  $p < 0.05$ ). Note the Y axis break in the ALP data.





**Figure 24.** Comparative responses of neonatal articular and growth plate chondrocytes, subject to continuous (aza cont) or transient (aza rec) 5-aza treatment, to BMP-2 administration. Expression of Col X and ALP mRNAs was assessed by qPCR. There was an approximately 400-fold difference between the levels of collagen type X mRNA expression in articular and growth plate chondrocytes (isolated from the same donor; upper panel) while ALP expression was over 30-fold higher in growth plate chondrocytes. Asterisks indicate a significant response to BMP-2 over the appropriate control sample. # indicate a significant effect of 5-aza over the appropriate control sample (ANOVA;  $p < 0.05$ )

## CHAPTER 6: CONCLUSIONS

The research presented in this thesis addressed the actions of 5-azacytidine on the chondrocytic phenotype in two contexts. The first series of experiments, presented in Chapter 3, focused on the suppression of collagen type II expression in osteoarthritic articular chondrocytes, and the responses of these cells to 5-aza administration. The second research focus addressed the phenotypic transitions that develop in osteoarthritic articular chondrocytes (Chapter 4) and the impact of 5-azacytidine on the phenotypic distinctions between articular and endochondral (growth plate) chondrocyte populations (Chapter 5).

The experiments addressing regulation of collagen type II expression demonstrated that 5-aza significantly increased collagen type II mRNA levels, and concurrently up-regulated a number of other critical matrix genes; however, a comprehensive analysis of the methylation status of the collagen type II promoter and enhancer CpG island sequences demonstrated comprehensively that the mechanism of 5-aza's effect on collagen type II expression was not direct CpG de-methylation in these regions. On the contrary, comparative analyses of control and 5-aza treated specimens indicated that 5-aza was not influencing the existing CpG methylation status at all. Adenoviral over-expression of p53 and p21, simulating the DNA damage repair response, did not alter collagen type II expression, suggesting that this potential mechanism was also not responsible for 5-aza's effects. Candidate gene screening identified TGF- $\beta$ 3 as a potential effector of 5-aza's activity, but additional experiments are necessary to determine whether TGF- $\beta$ 3 up-regulation is responsible for 5-aza's effect on

chondrocytes. Genome-wide methyl CpG screening of normal and osteoarthritic articular chondrocyte populations will provide unbiased data on the DNA methylation changes that occur in arthritis and could identify differentially regulated and expressed genes that contribute to disease progression. In these analyses, the issue of ‘age’ also needs to be addressed, since stochastic genomic methylation undoubtedly accumulates during the several decades required for the clinical development of osteoarthritis.

Although high throughput ChIP-chip and ChIP-SEQ technologies have revolutionized the capacity and resolution of epigenomic analyses, much of the existing data sets have been generated from cell lines and from a very limited number of normal tissues. There is a growing appreciation that the epigenetic profiles of cell lines do not reflect those of the parental tissues. This is particularly pertinent to the field of articular chondrocyte biology, since no phenotypically robust cell lines are available. In addition, it is now obvious that CpG methylation acts in concert with histone modifications and, in all likelihood, microRNAs to regulate cellular transcriptional activity.

In future experiments, primary cell specimens from multiple donors, representing both healthy and diseased states, should be used to generate a comprehensive and informative data base of normal and pathological epigenomes, encompassing a comprehensive panel of epigenetic marks, in representative tissues. Again, in the context of chondrocyte biology, this would be extremely challenging, since acquiring healthy articular cartilage from younger individuals (less than 30 years of age) is difficult. Appropriate non-rodent model species will need to be identified and validated to enable the development of a comprehensive cartilage epigenomic data base that can then be used to analyze and interpret human data sets.

The second research focus addressed the effects of 5-aza on the articular and endochondral chondrocytic phenotypes. This interest was prompted by several published studies that implicated 5-aza and, by extension, DNA methylation in the regulation of chondrocyte lineage commitment. Our own studies did not support the conclusion reached by other researchers that 5-aza is able to drive hypertrophic differentiation of articular chondrocytes, but growth plate differentiation was increased by transient 5-aza administration. As with the referenced published studies, the specific role of CpG methylation in this process was not determined. Ongoing research should focus on identifying the cellular mechanism(s) that mediate 5-aza's effects on chondrocyte hypertrophy. Given the critical influence cell cycle arrest has on the phenotypic transition into the hypertrophic phase of differentiation, the up-regulation of p53 and p21 by 5-aza is an obvious avenue for future investigation.

Accepting that the mechanisms of action that mediate 5-aza's effects on osteoarthritic and endochondral chondrocytes are currently uncertain, there are clear potential therapeutic applications for epigenetic modifying agents in arthritis and in endochondral bone formation. Several epigenetic agents have already been advocated for the suppression of catabolic enzymes that degrade cartilage matrix and our own data suggest that DNA methylation inhibitors could also support chondrocyte anabolic activities in the face of arthritic pathology.

## **AUTHOR'S BIOGRAPHY**

Evelyn Hasegawa Gonçalves Caporali was born in São Paulo, São Paulo, Brazil, on January 25, 1977. She graduated from Universidade Estadual Paulista “Julio de Mesquita Filho” in 1999 with a degree in Veterinary Medicine. In the same university, she finished the Small Animal Surgery Residency Program in 2003 and completed her Master's degree in Small Animal Surgery in 2004. She is currently doing a Small Animal Rotating Internship at Purdue University and will start a Small Animal Surgery Residency at University of Illinois at Urbana-Champaign in July 2011.